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## ERRATA

- Page 23, line 2 in SUMMARY, " $[\alpha]_{\text{D}}^{30}$ " should read " $[\alpha]_{\text{D}}^{22}$ " and " $(\text{CHCl}_3)$ " should read "water"
- Page 52, line 12 should read "Indoleacetic acid induced rooting at the cut surface sooner and more completely in"
- Page 52, line 16 should read "ethylene chlorohydrin caused them to root more readily at the cut surface when treated"
- Page 117, lines 13 and 14 should be deleted
- Page 210, line 7 from bottom, "solumn" should read "column"
- Page 210, line 6 from bottom, "ehown" should read "shown"
- Page 210, line 5 from bottom, "cral" should read "eral"
- Page 322, Table VIII, column 1, "Zealite" should read "Zeolite"

# INSECTICIDAL PROPERTIES OF EXTRACT OF MALE FERN (*ASPIDIUM FILIX-MAS* [L.] SW.)<sup>1</sup>

FRANK WILCOXON, ALBERT HARTZELL, AND FREDERICKA WILCOXON

## INTRODUCTION

The powdered rhizome of male fern (*Aspidium filix-mas* [L.] Sw.) has been used since ancient times as an anthelmintic. Investigation of the chemistry of the active principles appears to begin with the work of Luck (13) who isolated a crystalline substance described as "rhombische Blättchen" from the ether extract of the rhizome. Grabowski (9), Dacomo (8), and Schiff (14) also made chemical examinations of the extract, but the first comprehensive investigation is that of Boehm (1, 2, 3, 4, 5, 6) and Hausmann (11), who isolated a number of compounds which were shown to be derivatives of phloroglucinol propyl ketone in which the ring structures were linked through methylene groups. Treatment of the compounds with zinc dust and alkali led to the formation of methyl derivatives of phlorobutanone. Boehm, using the various compounds isolated by him, gives the results of toxicity tests on two species of frogs. These compounds varied greatly in their toxicity. For example, in the case of aspidin the lethal dose was 1 to 2 mg. injected into the lymph sac, while flavaspidic acid required 10 mg., and was lethal only after several hours.

Kraft (12) repeated some of the work of Boehm and concluded that the anthelmintic action was due to non-crystallizable acid material which had been overlooked by Boehm, and which constituted about 5 per cent of the extract.

There appear to be no records of tests on male fern extract or its constituents as a general insecticide, and the present paper deals with such tests on several insect species.

## MATERIALS AND METHODS

The extract used was a commercial product obtained from a reliable drug company. Crude filicin was obtained from this material by the method described by Boehm (1, p. 35-36). The commercial extract was ground in a mortar with magnesium oxide to give a grayish-green powder, and the latter was extracted repeatedly with water. The aqueous solution was acidified with sulphuric acid and the crude filicin obtained as a voluminous precipitate with a reddish tint, which was filtered, washed, and dried. The yield was 15 to 20 per cent of the weight of the extract. The "Filixsäure" of Boehm and other workers was isolated from crude filicin by dissolving

<sup>1</sup> Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 202.



in ether, adding acetone, and allowing to stand in the cold for several days. After repeated recrystallization from ether it was obtained as rhombic tablets of yellow color, and a melting point of  $184^{\circ}$  C. *Anal.* Calcd. for  $C_{35}H_{40}O_{12}$ : C, 64.38; H, 6.18. Found: C, 64.27, 64.15; H, 6.47, 6.63.

The species of insects used for the tests included mosquito larvae (*Culex quinquefasciatus* Say),<sup>2</sup> the bean aphid (*Aphis rumicis* L.), and house flies (*Musca domestica* L.). The tests on mosquito larvae were performed essentially as described by Campbell, Sullivan, and Smith (7). The test solution was made up by dissolving the material in 1.5 cc. of acetone, diluting to 1 liter, and subsequent dilutions were made from this stock. The tests on *Aphis rumicis* were performed as described previously (10). The house fly tests were performed according to the Peet-Grady method by a commercial laboratory.<sup>3</sup>

### RESULTS

*Tests on mosquito larvae.* Preliminary tests on mosquito larvae showed that the commercial oleoresin of male fern was toxic. Seventy per cent of the larvae were killed by 6.25 p.p.m., while 25 p.p.m. killed 100 per cent.

The toxicity of crude filicin was compared with that of purified "Filixsäure" in another experiment. The results are shown in Figure 1, in which the per cent dead is plotted against concentration using logarithmic probability paper. The LD<sub>50</sub> for crude filicin was estimated as 11 p.p.m., while that for "Filixsäure" was 2.9 p.p.m. It appears, therefore, that the latter compound must be one of the important toxic constituents of crude filicin.

*Tests on bean aphid.* Penetrol at 0.5 per cent concentration was used as a wetting agent. The sprays contained in addition 0.03 per cent and 0.1 per cent crude filicin. The results are shown in Table I.

TABLE I  
TOXICITY OF CRUDE FILICIN TO APHIS RUMICIS; CONCENTRATION  
OF PENETROL 0.5 PER CENT

Concn. of filicin, per cent	Total No. insects counted	No. alive	No. dead	Per cent dead
0.1	140	1	139	99.3
0.1	194	7	187	96.4
Penetrol check	171	121	50	29.2
0.03	90	5	85	94.4
0.03	205	32	173	84.3
Penetrol check	157	63	94	59.2
Penetrol check	177	91	86	48.5

<sup>2</sup> Hatched from eggs furnished through the courtesy of C. H. Bradley of Orlando, Florida.

<sup>3</sup> The tests were performed through the courtesy of S. B. Penick & Co., New York, N. Y.



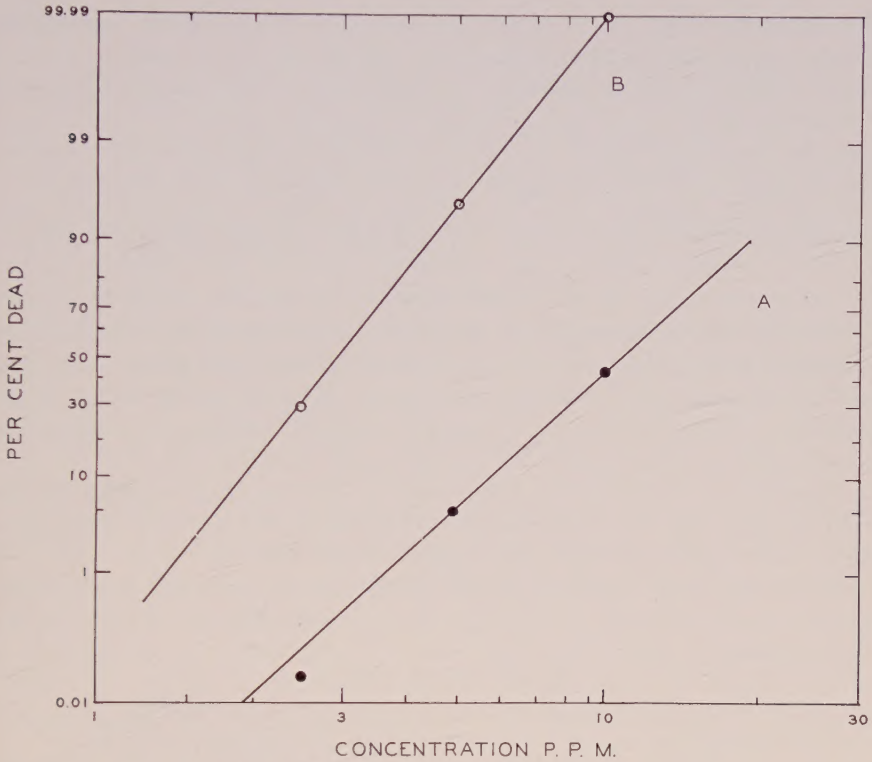


FIGURE 1. Comparative toxicity of crude filicin (A) and "Filixsäure" (B) to mosquito larvae.

*Tests on house flies.* The solution tested was made up to contain 0.2 per cent crude filicin and 0.05 per cent pyrethrins using "Deobase" as the solvent. The solution was compared with the official test insecticide. The results are shown in Table II. The grade of "B" was assigned to this sample as a result of these tests.

TABLE II  
TOXICITY OF PYRETHRUM-FILICIN MIXTURE TO HOUSE FLIES

Sample	Knockdown per cent	Kill per cent
0.05 per cent pyrethrins+0.2 per cent filicin	94.4	52.0
Official test insecticide	96.4	53.1

*Plant tolerance.* Limited tests have been performed on the following species of plants using a spray containing 0.5 per cent Penetrol and 0.05 per cent crude filicin. No injury was observed.

<i>Chrysanthemum</i> sp.	Tomato ( <i>Lycopersicon esculentum</i> Mill. var. Bonny Best)
African marigold ( <i>Tagetes erecta</i> L.)	Tree peony ( <i>Paeonia suffruticosa</i> Andr.)
Peach seedlings ( <i>Prunus persica</i> Sieb. & Zucc.)	<i>Verbena hybrida</i> Voss.
Rambler rose ( <i>Rosa</i> sp.)	<i>Verbena venosa</i> Gill. & Hook.
Snapdragon ( <i>Antirrhinum majus</i> L. var. Day Dream)	Zinnia ( <i>Zinnia elegans</i> Jacq. var. Giant Dahlia Flower)

## SUMMARY

The insecticidal properties of oleoresin of male fern have been tested on mosquito larvae, *Aphis rumicis*, and house flies. The "Filixsäure" of Boehm was found to be four times as toxic to mosquito larvae as crude filicin. Satisfactory control of *Aphis rumicis* was obtained with a spray containing 0.03 per cent of crude filicin, and 0.5 per cent Penetrol.

When tested by the Peet-Grady method on house flies a grade "B" insecticide was obtained with a spray containing 0.05 per cent pyrethrins and 0.2 per cent crude filicin in "Deobase" as a solvent.

No injury was observed when ten species of common plants were sprayed with a solution containing 0.05 per cent crude filicin and 0.5 per cent Penetrol.

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# AN ANALYSIS OF FACTORS CAUSING VARIATION IN SPORE GERMINATION TESTS OF FUNGICIDES.

## I. METHODS OF OBTAINING SPORES<sup>1</sup>

S. E. A. MCCALLAN AND FRANK WILCOXON

New fungicidal sprays and dusts are continually being developed, and it is a matter of considerable importance to be able to find out with the least expenditure of time and money whether these new preparations are better than those now in use. Large scale spraying experiments in the field are costly and time-consuming, and for this reason attempts have been made to test fungicides in the laboratory by observing their effects on the germination of spores of pathogenic fungi. Two criticisms commonly have been directed against the laboratory testing of fungicides; first that the tests are exceedingly variable, no two investigators obtaining the same results, even one investigator being unable to duplicate his own results at different times, and secondly that laboratory tests do not give a satisfactory indication of field performance. The first criticism must be answered before attempting the second.

Experiments accordingly have been designed to study the fundamental cause of variation in spore germination tests. This first paper will deal with the biological variation associated with the methods of isolating, producing, and germinating the spores. A second paper will discuss the mechanical errors introduced in techniques of spraying and regulating deposition.

### METHODS

The common slide-moist chamber method of testing (5, 7, 8) was employed in these studies. The brown-rot fungus, *Sclerotinia fruticola* (Wint.) Rehm., was selected for investigation because it is frequently used in laboratory tests and also because it is extremely variable and sensitive in its germination. The cultures were mass-spore isolates obtained from sweet cherries from three different localities in Suffolk County, Long Island, over a period of four years. For certain studies single-spore isolations were made from these cultures by the method of Ezekiel (2).

In the earlier tests (Tables I to IV) all spores were produced at 20° C. and germinated at room temperature. For all other tests the spores were produced and germinated in a constant temperature room designed solely for this purpose (Fig. 1). The mean temperature of the room was 21.0° C., with a standard deviation of 0.5° C. The spores were produced on potato-dextrose-agar slants (8 cc. of media per tube) and used for germination

<sup>1</sup> Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 203.  
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FIGURE 1. Constant temperature room for spore production and germination; maintained at  $21.0 \pm 0.5^{\circ}$  C.



tests when the cultures were seven days old. The spores were obtained for the earlier tests (Tables I to IV) by the customary method of washing from the agar slants as described on page 13. Counts of the spore concentration were made on three samples by means of a Fuchs-Rosenthal counting cell. The final concentration was adjusted to 50,000 spores per cc. with an average standard deviation of 5,600. Orange juice stimulant (8, 10) was used throughout. A constant source was assured for all tests, except the early ones, by filtering the juice of several oranges through USP IX collodion membranes, diluting to 5 per cent and placing in small stoppered vials held at below freezing temperatures until used. At first the concentration employed was 0.02 per cent; later as will be indicated this was changed to 0.10 per cent.

In order to eliminate spraying errors from this phase of the study, various concentrations of copper sulphate solutions were employed as the toxic agent. Copper sulphate and orange juice of a strength to give the final desired concentration were added together in large volumes. Two cc. of this solution were placed in a test tube and 0.5 cc. of spore suspension added. The mixture was well agitated and pipetted on to the glass slides giving drops of about 0.05 cc. in volume and of approximately equal areas.

Records on percentage germination were taken after 20 hours. Each observation was based on counts from two adjacent drops totaling 50 to 80 spores for the earlier tests, and 100 for the later ones. The percentage germination values were converted to equivalent angles according to the method of Bliss (1) to give greater precision for statistical analyses. The analysis of variance procedure was generally used since this enables one to isolate the variation due to the various factors and to ascribe to each factor its relative importance. In this paper the results on over 400,000 spores are analyzed.

#### DEFINITION OF TERMS

Specific terms employed in this paper are defined as follows:

*Field isolate.* A mass-spore isolation of a given fungus obtained from a different field station or the same station at a different year.

*Single-spore isolate.* A single-spore isolation made from a field isolate.

*Stock slant.* An individual test tube culture, either of a field or single-spore isolate, from which transfers are made.

*Replicate transfers.* Transfers made from the same stock slant at the same time and grown under the same conditions.

*Replicate counts.* Replicate counts made on spores from the same replicate transfer exposed under the same conditions, at the same time, to the same concentration of a toxic agent. They may be on the same or different slides and in the same or different moist chambers.

*Replicate experiments.* Tests replicated at different times and hence

with different lots of spores, though of the same isolate, and possibly from the same stock slant.

*Significance. Highly significant*—Odds greater than 100:1. *Significant*—Odds between 100:1 and 20:1. *Not significant*—Odds less than 20:1, (9).

## RESULTS

### REPLICATE TRANSFERS

Tests were made on the consistency of germination of spores from different test tube slants transferred from the same stock slant at the same time and produced and germinated under presumably the same conditions. The results of such a comparison are shown in Table I.

TABLE I

PERCENTAGE GERMINATION OF *SCLEROTINIA FRUCTICOLA* CONIDIA IN COPPER SULPHATE SOLUTIONS. SPORES FROM DIFFERENT REPLICATE TRANSFERS AND STOCK SLANTS. DUPLICATE COUNTS ON 65-75 SPORES

P.p.m. Cu	Stock slant	Replicate transfers					
		1		2		3	
0.125	1	75.8	85.7	68.3	78.7	85.5	83.3
	2	92.7	81.2	84.1	79.2	62.3	30.0
	3	90.0	74.3	85.8	66.2	60.0	45.6
0.250	1	27.1	52.8	84.6	91.1	95.4	93.2
	2	97.0	98.6	98.6	97.2	97.2	93.8
	3	95.3	82.8	92.8	84.8	95.7	95.3
0.500	1	26.1	31.8	52.3	46.3	67.2	76.1
	2	80.0	74.3	93.8	96.8	16.7	24.6
	3	60.7	30.3	52.8	63.3	63.1	67.2

### Analysis of Variance

	Degrees of freedom	Sums of squares	Variance
Replicate counts	27	989	37
Replicate transfers	6	3704	617
Stock slants	2	586	293
Cu concentrations	2	4383	2191
Replicate transfers × concentrations	12	2757	230
Stock slants × concentrations	4	919	230
Total	53	13338	

Replicate counts vs. Replicate transfers    Highly significant  
Replicate transfers vs. Stock slants        Not significant

If the only sources of variation were those of random sampling, the mean square or variance, when the results are expressed as equivalent angles would be expected to be 25 on the basis of 100 spores per count, or 50 on 50 spores. It will be seen in Table I that replicate counts are subject

only to the errors of random sampling which confirms previous results (7). However, replicate transfers vary greatly from one another, as much indeed as replicate transfers from different stock slants. If spores produced and germinated at the same time under supposedly the same conditions will not respond alike, spores produced and germinated at different times cannot be expected to act alike. The problem, therefore, becomes that of determining the cause of the variation among replicate transfers.

*Cleanliness of moist chambers.* The importance of chemically clean moist chambers in spore germination studies is generally recognized and has been stressed before (5). However, it is necessary that the chambers be freshly washed just before use in order to eliminate any material that they may have collected or adsorbed while standing. In a test in which spores were germinated in freshly washed chambers and chambers which had been washed and stored for several months, it was found that the variation of the spore germination in the stored chambers was significantly greater than that of the fresh chambers. Thereafter all chambers were freshly washed in chromic acid cleaning solution or soap and water not longer than one week before use.

#### SIZE OF TEST TUBES

The variation in size of test tubes used for cultures might be a possible source of variation in the spore germination of replicate transfers. Small tubes of 13 mm. inside diameter and large tubes of 18 mm., were selected, potato dextrose agar added, and the tubes similarly plugged and slanted.

The results given in Table II show that the variation produced by the different sized tubes was not significantly greater than that of replicate transfers of the same tube size. However, in order to give standard conditions tubes of 17 mm. inside diameter were used thereafter.

TABLE II

CONIDIA OF *SCLEROTINIA FRUCTICOLA* PRODUCED IN SMALL AND LARGE CULTURE TUBES. THREE REPLICATE TRANSFERS FOR EACH TUBE SIZE. DUPLICATE COUNTS ON PERCENTAGE GERMINATION OF 70-80 SPORES IN COPPER SULPHATE SOLUTIONS

P.p.m. Cu	Small tubes			Large tubes		
	1	2	3	4	5	6
0.125	100.0 98.7	98.8 91.8	97.5 100.0	85.9 89.4	97.6 98.6	85.4 93.4
0.250	97.4 98.5	97.0 90.9	94.4 98.4	42.9 79.4	96.3 91.9	83.9 91.6
0.500	100.0 76.5	98.6 98.4	100.0 98.0	16.7 15.6	93.4 93.0	16.4 16.7
0.750	49.4 59.0	94.4 97.2	85.3 89.6	15.2 14.2	71.4 68.6	13.6 12.8

TABLE II (Cont'd.)—Analysis of Variance

	Degrees of freedom	Sums of squares	Variance
Replicate counts	24	992	41
Replicate transfers	4	4596	1149
Tube sizes	1	6697	6697
Cu concentrations	3	6115	2038
Replicate transfers $\times$ concentrations	12	2555	213
Tube sizes $\times$ concentrations	3	1973	657
Total	47	22928	
Replicate counts vs. Replicate transfers	Highly significant		
Replicate transfers vs. Tube sizes	Not significant		

## TIGHTNESS OF COTTON PLUGS

In some species of fungi it is considered that conidial production is dependent to some extent on the relative humidity which in turn may be controlled by the tightness of the cotton plugs. Hence it is possible that the germination response of spores from tubes differently plugged might also vary. Tubes of the same size and containing the same amount of agar, similarly slanted, were stoppered with loose, medium, and tight cotton plugs. There was no consistent difference in the appearance of the cultures or amount of spore production. Weights taken daily on sterile tube indicated no difference in water loss.

The results of germination tests are given in Table III and indicate that differences in tightness of cotton plugs will not account for the variation in replicate transfers.

## KIND OF INOCULUM

In making transfers of cultures it is possible to transfer either spores

TABLE III

CONIDIA OF *SCLEROTINIA FRUCTICOLA* PRODUCED IN CULTURE TUBES WITH LOOSE, MEDIUM, AND TIGHT COTTON PLUGS. FOUR REPLICATE TRANSFERS FOR EACH SIZE OF PLUG. DUPLICATE COUNTS ON PERCENTAGE GERMINATION OF 50-60 SPORES IN COPPER SULPHATE SOLUTIONS

P.p.m. Cu	Loose plugs				Medium plugs				Tight plugs			
	1	2	3	4	5	6	7	8	9	10	11	12
0.250	100.0	100.0	92.5	97.0	88.3	94.6	95.4	91.9	96.5	98.5	100.0	97.0
	100.0	94.6	98.1	96.2	79.0	98.0	93.9	98.0	86.0	85.3	98.0	96.2
0.500	100.0	100.0	29.0	96.8	5.2	95.2	68.7	94.6	91.6	92.0	95.0	85.5
	98.8	96.4	31.0	97.1	1.5	100.0	77.9	94.8	83.4	94.0	92.1	59.4
1.250	94.8	26.1	0	70.5	0	75.0	0	64.8	13.2	15.0	0	14.2
	93.9	7.4	0	52.9	3.4	64.8	2.3	61.4	18.5	13.2	15.2	13.6



TABLE III (Cont'd.)—Analysis of Variance

	Degrees of freedom	Sums of squares	Variance
Replicate counts	36	1378	38
Replicate transfers	9	15344	1705
Size of plugs	2	1309	655
Cu concentrations	2	31796	15898
Replicate transfers × concentrations	18	7864	437
Plugs × concentrations	4	1213	
Total	71	58904	
Replicate counts vs. Replicate transfers    Highly significant			
Replicate transfers vs. Size of plugs        Not significant			

alone or agar blocks containing mycelium, or a mixture of the two. Two sets of agar slants were inoculated respectively with spores alone and agar blocks of mycelium both obtained from the same stock slant.

The results are reported in Table IV. It will be seen that the variation due to different sources of inoculum is not significantly greater than that

TABLE IV

SCLEROTINIA FRUCTICOLA CONIDIA PRODUCED FROM INOCULUM OF SPORES AND OF AGAR BLOCKS OF MYCELIUM FROM THE SAME STOCK SLANT. THREE DIFFERENT FIELD ISOLATES AND TWO REPLICATE TRANSFERS FOR EACH KIND OF INOCULUM. TRIPPLICATE COUNTS OF PERCENTAGE GERMINATION IN COPPER SULPHATE SOLUTIONS ON 80-100 SPORES

P.p.m. Cu	Field isolate	Inoculum of spores		Inoculum of mycelium	
		1	2	3	4
0.50	V 35	100.0	95.4	67.9	94.4
		97.5	92.2	94.9	87.9
		98.9	97.7	98.7	93.4
	V 37	93.5	74.7	92.9	85.0
		93.9	71.0	98.7	89.3
		96.0	82.1	95.5	97.4
	L 37	86.8	93.4	97.6	87.8
		92.0	96.4	90.7	50.0
		89.0	91.0	100.0	91.5
1.25	V 35	94.7	47.3	72.0	51.5
		94.8	98.1	52.4	80.5
		95.1	92.9	62.4	75.4
	V 37	54.0	33.0	71.9	91.7
		50.5	26.0	71.0	89.6
		57.0	31.4	90.0	92.7
	L 37	56.8	69.7	15.2	29.3
		18.5	70.0	20.1	20.4
		22.2	91.6	22.0	38.0

TABLE IV (Cont'd.)—Analysis of Variance

	Degrees of freedom	Sums of squares	Variance
Replicate counts	48	3214	67
Replicate transfers	6	1849	308
Kind of inoculum	1	70	70
Field isolates	2	2451	1225
Cu concentrations	1	8778	8778
Replicate transfers $\times$ concentrations	6	1116	186
Residue	7	5796	828
Total	71	23274	
Replicate counts vs. Replicate transfers	Highly significant		
Replicate transfers vs. Kind of inoculum	Not significant		
Replicate transfers vs. Field isolates	Not significant		

of replicate counts or random sampling. The variation among replicate transfers remains very high.

#### MASS-SPORE VS. SINGLE-SPORE ISOLATES

It is generally accepted that single-spore isolates are more uniform than mass-spore isolates. Hansen and Smith (3) have shown that *Botrytis cinerea* is multinucleate and exists in a number of more or less dissimilar morphological strains. When single-spore isolations are repeated through a number of successive generations some of the strains remain constant, others continue to break up into further variations. Heuberger (4) has demonstrated that the conidia of *Sclerotinia fructicola* are multinucleate, which has been confirmed by Dr. Eckerson of this Institute. It was thought that the variation in germination of conidia of *Sclerotinia fructicola* might be due to nuclear heterogeneity. A number of single-spore isolations were made from the various field isolates of *S. fructicola* by the method of Ezekiel (2). From these, repeated single-spore isolations were made, thus giving a large number of second generation single-spore isolates. No constant morphological characters appeared in any of the isolates nor was there any evidence that the various field isolates were different biological strains.

Spore germination tests were performed with a number of the second generation single-spore isolates. The variance for replicate transfers of the single-spore cultures in these tests was compared with that of a number of mass-spore cultures. The data are summarized below.

	Degrees of freedom	Sums of squares	Variance
Mass-spore	39	39412	1011
Single-spore	34	28125	827

There is no significant difference between the variation of replicate transfers from mass or single-spore cultures. Further comparisons on mass and single-spore isolates will appear on page 16.



TABLE VI

PERCENTAGE GERMINATION OF CONIDIA OF *SCLEROTINIA FRUCTICOLA* IN COPPER SULPHATE SOLUTIONS. SPORES OBTAINED BY RUBBING AND CENTRIFUGING TECHNIQUE. FOUR REPLICATE TRANSFERS.\* SIX FIELD ISOLATES. THREE REPLICATE EXPERIMENTS. 100 SPORES PER COUNT

P.p.m. Cu	Exp. No.	Field isolate					
		V 35	V 37	V 38	L 37	L 38	S 38
0.5	1	69* 85 61 85	84 82 88 75	91 71 88 92	88 91 70 85	82 82 77 88	77 91 77 88
	2	77 83 82 89	91 68 82 92	90 84 86 90	85 76 84 84	84 77 80 86	76 64 66 85
	3	95 89 92 97	69 82 78 55	91 95 92 89	97 95 92 95	89 83 86 87	89 90 95 93
1.0	1	57 42 42 43	62 53 70 75	53 40 80 52	53 72 54 57	76 70 75 73	45 79 50 63
	2	49 51 41 64	83 49 57 49	61 71 60 54	65 56 58 71	78 66 59 70	53 41 32 38
	3	78 68 66 90	55 71 51 49	79 57 75 82	85 81 74 91	65 77 50 69	68 72 76 67
2.0	1	8 8 16 3	27 25 13 16	53 39 51 20	16 11 16 13	31 28 53 13	31 23 39 30
	2	28 34 11 44	32 37 31 34	20 20 17 23	9 11 8 12	12 4 16 5	15 24 50 27
	3	63 43 24 31	18 22 23 28	12 20 26 10	28 17 29 16	24 20 31 67	27 24 21 19
3.0	1	2 0 2 1	18 26 9 10	29 22 18 7	5 0 1 1	3 13 3 2	6 5 16 7
	2	4 7 4 20	5 4 9 2	3 1 3 1	6 3 2 4	3 0 0 3	4 5 7 1
	3	14 17 14 11	10 7 19 8	5 2 4 1	15 4 5 17	2 3 13 10	8 6 9 8

## Analysis of Variance

	Degrees of freedom	Sums of squares	Variance
Replicate transfers	54	2163.7	40.07
Field isolates	5	128.3	25.66
Replicate experiments	2	1702.7	851.35
Cu concentrations	3	121756.6	40585.53
Replicate transfers × concentrations	162	5354.9	33.05
Field isolates × experiments	10	3666.4	366.64
Field isolates × concentrations	15	1726.8	115.12
Experiments × concentrations	6	302.0	50.33
Isolates × experiments × concentrations	30	3009.3	100.31
Total	287	139810.7	

Replicate transfers vs. Field isolates Not significant  
 Replicate transfers vs. Replicate experiments Significant

\* Results on same replicate transfer are reported in same quarter of box e.g., results for first replicate transfer of V 35 Experiment 1 are 69, 57, 8, and 2.



TABLE VII  
PERCENTAGE GERMINATION OF CONIDIA OF SCLEROTINIA FRUCTICOLA IN COPPER SULPHATE SOLUTIONS. SPORES OBTAINED BY RUBBING AND CENTRIFUGING TECHNIQUE. SIX SINGLE-SPORE ISOLATES FROM FIELD ISOLATE V 35. FOUR REPLICATE TRANSFERS.\* THREE REPLICATE EXPERIMENTS. 100 SPORES PER COUNT

P.p.m. Cu	Exp. No.	Single-spore isolates									
		DA		DK		EE		EL		FD	
0.5	1	88* 87 94 91	81 86 91 93	82 88 88 78	92 86 65 75	68 70 71 72	74 71 58 61				
	2	86 87 70 87	89 93 86 80	92 73 87 94	82 84 92 91	82 85 89 84	94 87 94 78				
	3	89 87 90 79	74 80 67 87	88 79 80 89	73 84 80 86	81 83 85 83	93 94 80 92				
1.0	1	69 43 76 69	70 61 57 38	51 68 45 57	43 36 47 50	55 45 47 26	60 71 64 36				
	2	57 62 38 73	62 87 66 53	72 66 61 76	70 58 80 73	70 61 52 59	75 60 79 66				
	3	83 79 80 56	73 42 45 58	79 72 59 67	52 65 48 78	67 62 58 61	66 77 67 70				
2.0	1	27 47 33 42	12 9 13 3	50 21 16 26	12 22 6 7	38 17 35 21	41 18 21 24				
	2	28 26 29 23	26 30 26 22	25 54 34 44	25 24 30 42	53 33 32 43	53 25 29 12				
	3	35 49 33 35	43 38 48 62	25 30 18 17	22 34 31 38	19 22 12 25	34 19 23 21				
3.0	1	8 6 13 10	2 0 2 1	7 1 4 2	2 4 1 0	2 1 7 0	8 17 11 6				
	2	4 8 12 6	10 18 13 13	16 25 7 20	13 6 6 3	22 20 13 16	19 6 12 4				
	3	19 11 18 6	28 19 40 24	8 5 7 8	13 14 28 29	8 7 5 10	13 16 4 19				

Analysis of Variance

	Degrees of freedom	Sums of squares	Variance
Replicate transfers	54	2988.9	55.35
Single-spore isolates	5	618.9	123.78
Replicate experiments	2	2829.4	1414.70
Cu concentrations	3	100703.8	33567.93
Replicate transfers×concentrations	162	4038.1	24.92
Single-spore isolates×experiments	10	1922.4	192.22
Single-spore isolates×concentrations	15	477.5	31.83
Experiments×concentrations	6	454.3	75.72
Isolates×experiments×concentrations	30	3687.4	122.91
Total	287	117720.7	

Replicate transfers vs. Single-spore isolates Not significant  
Replicate transfers vs. Replicate experiments Highly significant

\* See footnote Table VI.

man, care being taken not to break the surface of the media. Spores were filtered through cheesecloth, centrifuged as above, and germinated in 0.10 per cent orange juice.

A total of 31 experiments on the various techniques with single and mass-spore isolates were performed and about 300,000 spores counted. The results are summarized in Table V. Actual examples of the variation resulting from the washing and not centrifuging technique may be seen in Tables I, II, III, and IV, and from the rubbing and centrifuging in Tables VI and VII.

It will be seen in Table V that by improvements in the technique of obtaining spores, whereby the soluble nutrients have been removed, a great reduction has been effected in the variation of replicate transfers. By changing from the original technique to that of rubbing and centrifuging there has been a 15 to 20-fold reduction in variation thus leaving a residual error comparable to that of random sampling.

The rubbing technique while not showing a significant difference in precision from the vacuum technique is considered more desirable because of its greater simplicity. The act of centrifuging alone, as shown in the washing technique, brought about a great reduction in variation. It will be noted that the increase in concentration of orange juice has not affected the variation of replicate transfers. Even with the improved technique there is no significant difference in variation between mass and single-spore isolates.

#### VARIATION OF REPLICATE EXPERIMENTS WITH MASS AND SINGLE-SPORE ISOLATES

Tests were made of the variation of replicate experiments using both mass and single-spore isolates. This procedure will also measure the individuality or consistency of germination of different isolates through replicate experiments. Two isolates might appear to be different in one experiment but unless this difference is maintained in different experiments it cannot be considered significant.

The improved technique of obtaining spores by rubbing and centrifuging was employed. Since it has been shown in this paper that the replicate counts do not vary appreciably more than is to be expected by random sampling, this practice was discontinued and a single count on 100 spores recorded. Four replicate transfers were used throughout at concentrations of 0.5, 1.0, 2.0, and 3.0 p.p.m. copper. Typical examples of replicate experiments on the same isolates are shown for mass-spore cultures in Table VI, and for single-spore in Table VII.

A summary of all the analyses on the replicate experiments is given in Tables VIII and IX.

TABLE VIII

SUMMARY OF DATA ON MASS-SPORE ISOLATES WITH RUBBING AND CENTRIFUGING  
TECHNIQUE OF OBTAINING SPORES

	Degrees of freedom	Sums of squares	Variance
Replicate transfers	66	2547	38.6
Replicate experiments	3	1938	612.7
Field isolates	6	165	27.5
Cu concentrations	6	135567	22594.5
Replicate transfers $\times$ concentrations	198	7662	38.7
Field isolates $\times$ replicate experiments	11	4062	369.3
Other interactions	60	6501	108.3
Total	350	158442	
<hr/>			
Replicate transfers vs. Replicate experiments	Highly significant		
Replicate transfers vs. Field isolates	Not significant		

TABLE IX

SUMMARY OF DATA ON SINGLE-SPORE ISOLATES WITH RUBBING AND CENTRIFUGING  
TECHNIQUE OF OBTAINING SPORES

	Degrees of freedom	Sums of squares	Variance
Replicate transfers	239	13435	56.2
Replicate experiments	8	11041	1380.1
Field isolates	7	6952	993.1
Single-spore isolates within field isolates	24	5811	242.1
Cu concentrations	18	465279	25848.8
Replicate transfers $\times$ concentrations	717	20306	28.3
Field isolates $\times$ replicate experiments	7	2722	388.9
Single-spore isolates $\times$ replicate experiments	34	5291	155.6
Other interactions	240	21988	
Total	1294	552825	
<hr/>			
Replicate transfers vs. Replicate experiments	Highly significant		
Field isolates vs. Field isolates $\times$ repl. exps.	Not significant		
Single-spore isolates vs. Single-spore isol. $\times$ repl. exps.	Not significant		

It will be noted that the variation of replicate transfers is almost reduced to that of random sampling. The difference probably may be ascribed to the variation of spore concentrations. However, despite the low variation of replicate transfers, the variation of replicate experiments remains high. A possible explanation for this is that the sterile slants inoculated to produce spores for the different experiments may be from different batches of agar and even where these were the same the slants would differ in age at the different experiments. Until such time as this source of variation can be substantially reduced our criterion for the comparison of isolates must be the consistency of the difference in different experiments, that is the interaction isolates  $\times$  experiments. Likewise in evaluating fun-

gicides our criterion for error must be the interaction fungicides  $\times$  experiments.

The different field isolates as represented by mass-spore cultures in Table VIII show no significant difference from one another. Since one of these isolates, V 35, has been in culture for four years it is also an indication that there has been no loss or change in spore-germinating capacity through repeated culturing on artificial media.

The data of Table IX on cultures from second generation single-spore isolates indicate that while the field and single-spore isolates vary more than replicate transfers of the same isolate, or than error (concentration—replicate transfer interaction) nevertheless they are not significant over their respective experiment interactions. We must therefore conclude that neither the single-spore isolates nor the field isolates show any consistent individuality.

#### DISCUSSION

The chief sources of variation in spore germination tests of fungicides are likely to be (a) improperly cleaned glassware, especially slides and moist chambers; (b) age of cultures; (c) concentration of spore suspension; (d) temperature; (e) introduction of variable stimulants from culture media; (f) experimental or day-to-day error due to use of different lots of spores; and (g) errors of concentration in fungicide. The first two factors can be readily controlled and the last will be elaborated on in detail in the second paper of this series.

The concentration of the spore suspension should be regulated as far as possible since the toxicity of a given compound is likely to decrease with increasing numbers of spores (6, 7). The ability to obtain uniform samples of spore suspension is dependent chiefly on the properties of the spores themselves. In the case of *Sclerotinia fructicola* the coefficient of variation for successive counts in samples of the same lot, is about 17 per cent. This variation will tend to make the residual error somewhat greater than that of pure random sampling.

If possible the tests should be performed in a constant temperature room or chamber. Many "lost" experiments may be attributed to fluctuating temperatures. Since the acquisition of the constant temperature room over a million spores have been counted during a period of 18 months without the loss of a single experiment. Controls of *Sclerotinia fructicola* usually considered very variable have germinated consistently between 96 and 100 per cent.

By the use of a suitable technique, such as rubbing and centrifuging, it is possible to eliminate practically all of the variation due to stimulants from the substrate. Any residual variation over and above that of random sampling, if necessary, can be further reduced by mixing together spores



from several replicate transfers. It has been observed that when the spores of *Sclerotinia fructicola* and various other species are washed and centrifuged or otherwise separated from soluble nutrients of the media their germination is reduced. It is doubtful if these species will germinate readily in pure distilled water. The use of added stimulants such as orange juice may be questioned. Nevertheless it appears more desirable to remove the uncertain and variable stimulants from the culture medium and replace them by the more constant orange juice.

As the various factors responsible for variation are reduced or eliminated so the day-to-day variation will be diminished. However, it is problematical if this day-to-day error can ever be reduced to that of random sampling. It therefore becomes necessary to compare fungicides on the basis of the consistency of their differences in different experiments. This also may be accomplished by running a standard fungicide in every experiment with which the other materials may be compared (11).

#### SUMMARY

1. An analysis of the biological factors causing variation in spore germination tests of fungicides was attempted. Studies were made of the germination of about 400,000 conidia of *Sclerotinia fructicola* in copper sulphate solutions, using the slide-moist chamber technique. The spores were obtained from seven-day-old cultures on potato-dextrose-agar slants. For the most part, the spores were produced and germinated in a constant temperature room designed for this purpose and maintained at  $21.0 \pm 0.5^{\circ}\text{C}$ .

2. It was found that spores from replicate transfers, i.e., similar agar slants inoculated at the same time from the same stock culture, vary a great deal more than would be expected from random sampling. Replicate counts, however, on spores from the same transfer vary no more than random sampling.

3. This variation is not due to differences in the size of culture test tubes, tightness of cotton plugs, or of inoculum consisting of spores or of mycelium.

4. By improving the technique of obtaining the spores from the agar slants a 15 to 20-fold reduction was effected in the variation of replicate transfers, making it comparable to the unavoidable error of random sampling. The improved technique consists of adding distilled water to the culture tube, lightly rubbing the spores off with a rubber policeman, filtering the spore suspension through cheesecloth, centrifuging and decanting the supernatant liquid containing water-soluble nutrients from the agar slants which are responsible for the variation.

5. Detailed comparisons of mass-spore isolates from several localities and of single-spore isolates obtained from them show no consistent differences either between or among the various mass or single-spore isolates

as to variability of replicate transfers or response to given concentrations of copper.

6. Even with the use of the improved technique there remains a significant variation between similar experiments repeated at different times. This variation is possibly due in part to the necessity of using different batches or ages of agar to produce spores for the different experiments.

7. Until the variation of replicate experiments, due to the use of different lots of spores, can be reduced, our criterion for comparing fungicides must be the consistency of their differences in different experiments.

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## PROPERTIES OF CRYSTALLINE $\beta$ -2-CHLORO-ETHYL-*D*-GLUCOSIDE<sup>1</sup>

JACK COMPTON

The condensation of *D*-glucose with ethylene chlorohydrin in the presence of hydrogen chloride to yield an uncrystallizable mixture of  $\alpha$ - and  $\beta$ -2-chloroethyl-*D*-glucoside was first reported by Schroeter and Strassberger (7). In view of the recent interest shown in the halogeno-alkyl glucosides (a) as intermediary substances in the preparation of choline glucosides (4), and (b) as a means of studying induced glucoside formation in plants (5, 6), an attempt was made to obtain 2-chloroethyl-*D*-glucoside in crystalline form.

Employing conditions similar to those described by Jackson (4), Coles, Dodds, and Bergeim (1), and by Miller (5), crystalline  $\beta$ -tetraacetyl-2-chloroethyl-*D*-glucoside was obtained which upon careful deacetylation (3) yielded crystalline  $\beta$ -2-chloroethyl-*D*-glucoside with a melting point of 70°–71° C. and specific rotation in water,  $-29.0^\circ$ . Catalytic hydrogenation of  $\beta$ -2-chloroethyl-*D*-glucoside in the presence of alkali and Raney nickel catalyst resulted in the formation of  $\beta$ -ethyl-*D*-glucoside which was isolated as the crystalline tetraacetate.

$\beta$ -2-chloroethyl-*D*-glucoside is the first of the halogeno-alkyl glycosides to be obtained in crystalline condition.

### EXPERIMENTAL

#### PREPARATION OF $\beta$ -TETRAACETYL-2-CHLOROETHYL-*D*-GLUCOSIDE

With slight modification  $\beta$ -tetraacetyl-2-chloroethyl-*D*-glucoside was prepared according to the procedure described by Jackson (4).

To a solution of 45 g. of acetobromoglucose and 134 g. of ethylene chlorohydrin in 312 cc. of dry benzene, 46 g. of dry silver carbonate were added in small portions over a period of 10–15 minutes with vigorous stirring. The mixture was cooled with ice-water during the first 15–20 minutes to control the temperature resulting from the reaction. Finally the mixture was stirred at room temperature until a negative test for ionic halogen was obtained. After filtration and thorough washing with water the benzene solution was dried over anhydrous sodium sulphate, filtered, and concentrated under higher vacuum to a solid crystalline mass. The product was then recrystallized from absolute ethyl alcohol. Upon concentrating the mother liquors from the first recrystallization additional pure material was obtained. Yield, 36 g. or 80 per cent of theoretical. Melting point, 118°–119° C.,  $[\alpha]_D^{30}$ ,  $-13.0^\circ$  (c, 4.087, U.S.P.  $\text{CHCl}_3$ ).

<sup>1</sup> Cellulose Department, Chemical Foundation, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York.

Jackson (4) reported a yield of 69 per cent; melting point,  $118.5^{\circ}$ – $119.5^{\circ}$  C., and  $[\alpha]_D, -13.7^{\circ}$  ( $\text{CHCl}_3$ ).

#### CRYSTALLINE $\beta$ -2-CHLOROETHYL-*d*-GLUCOSIDE

To a solution of 5 g. of  $\beta$ -tetraacetyl-2-chloroethyl-*d*-glucoside dissolved in 160 cc. of anhydrous methyl alcohol there were added at  $0^{\circ}$  C. 5 cc. of a 0.4 N solution of barium methylate in methyl alcohol (3). After standing at  $5^{\circ}$  C. for 20 hours the barium methylate was decomposed by the addition of the exact equivalent of 0.5 N sulphuric acid solution. The solution was then treated with charcoal, filtered through Celite, and concentrated under diminished pressure to a thick sirup. The crude product was further purified by dissolving in hot ethyl acetate, treating with charcoal, and again filtering through Celite. The clear filtrate was then concentrated under diminished pressure to a thick sirup and dissolved in 10 cc. of ethyl acetate. The first crystals were obtained by allowing the solution to stand for several months at  $5^{\circ}$  C., but in subsequent experiments it was only necessary to nucleate the ethyl acetate solutions at this point to initiate crystallization. After standing overnight at  $5^{\circ}$  C. the product had completely crystallized in the form of needle clusters. Yield, 3 grams. After the second recrystallization from ethyl acetate-petroleum ether a constant melting point of  $70^{\circ}$ – $71^{\circ}$  C. was obtained,  $[\alpha]_D^{22}, -29.0^{\circ}$  (c. 3.244, water). *Anal.* Calcd. for  $\text{C}_8\text{H}_{15}\text{O}_6\text{Cl}$ : C, 39.57; H, 6.23; Cl, 14.61. Found C, 39.19; H, 6.00; Cl, 14.66.

#### PREPARATION OF $\beta$ -TETRAACETYL-2-CHLOROETHYL-*d*-GLUCOSIDE BY THE ACETYLATION OF $\beta$ -2-CHLOROETHYL-*d*-GLUCOSIDE

$\beta$ -2-chloroethyl-*d*-glucoside (229 mg.) was dissolved in 3 cc. of dry pyridine and 1 cc. of acetic anhydride added. After thorough stirring the mixture was allowed to stand overnight at room temperature and then poured with stirring into 100 cc. of ice-water. The crystalline material separating was removed by filtration, thoroughly washed with cold water and dried. Yield, 343 mg. After one recrystallization from ethyl alcohol a melting point of  $118^{\circ}$ – $119^{\circ}$  C. was obtained unchanged by further recrystallization;  $[\alpha]_D^{30}, -13.0^{\circ}$  (c. 4.144,  $\text{CHCl}_3$ ). A mixed melting point of this material with the original  $\beta$ -tetraacetyl-2-chloroethyl-*d*-glucoside showed no depression.

#### CATALYTIC REDUCTION OF $\beta$ -2-CHLOROETHYL-*d*-GLUCO- SIDE FOLLOWED BY ACETYLATION TO YIELD $\beta$ - TETRAACETYLETHYL-*d*-GLUCOSIDE

To a solution of 2.27 g. of  $\beta$ -2-chloroethyl-*d*-glucoside in 75 cc. of ethyl alcohol, 4 cc. of 18 per cent sodium hydroxide solution and a suspension of Raney nickel catalyst in ethyl alcohol were added. The mixture was then



subjected to 3 atmospheres hydrogen pressure and hydrogenation allowed to proceed until the theoretical amount of hydrogen had been absorbed. The nickel catalyst was then removed by filtration and the excess alkali neutralized by passing in a stream of carbon dioxide gas. The separating salts were removed by filtration and the clear filtrate concentrated under diminished pressure to a thick sirup. The glucoside was dissolved in hot acetone, the solution filtered and concentrated under diminished pressure. The sirup was finally dissolved in 10 cc. of dry pyridine and 5 cc. of acetic anhydride added. After standing overnight at room temperature the solution was poured into 100 cc. of ice-water. The sirupy product first separating soon crystallized and was removed by filtration. After thorough washing with cold water the air-dried material was recrystallized from absolute ethyl alcohol. Yield, 1.4 grams. Melting point,  $106^{\circ}$ – $107^{\circ}$  C. A mixed melting point of this material with an authentic specimen (2) of  $\beta$ -tetraacetyl-ethyl-*D*-glucoside showed no depression.

#### SUMMARY

$\beta$ -2-chloroethyl-*D*-glucoside has been obtained in crystalline form with melting point  $70^{\circ}$ – $71^{\circ}$  C.,  $[\alpha]_D^{30}$ ,  $-29.0^{\circ}$  ( $\text{CHCl}_3$ ). Hydrogenation of the glucoside in the presence of Raney nickel catalyst followed by acetylation yields crystalline  $\beta$ -tetraacetylethyl-*D*-glucoside.

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## SYNTHESIS OF $\beta$ -*o*-CHLOROPHENOL-*d*-GLUCOSIDE<sup>1</sup>

LAWRENCE P. MILLER

When potato tubers or gladiolus corms are treated with ethylene chlorohydrin in order to break the rest period (2, 3, 4), the absorbed ethylene chlorohydrin is converted into  $\beta$ -(2-chloroethyl)-*d*-glucoside (7, 9). Further experiments with other plant tissues and with other aglucons have indicated that the formation of glycosides from absorbed non-naturally occurring substances can take place quite generally among the higher plants (8). Thus if gladiolus corms are exposed to the vapor of *o*-chlorophenol, considerable quantities are taken in by the corms, and several days after the end of the period of exposure the *o*-chlorophenol is no longer recoverable by distillation unless tissue extracts are first acted upon by emulsin (unpublished). Since it had been found that  $\beta$ -(2-chloroethyl)-*d*-glucoside is formed by gladiolus corms from ethylene chlorohydrin, it was reasonable to suppose that in all probability the glycoside formed from *o*-chlorophenol would be  $\beta$ -*o*-chlorophenol-*d*-glucoside. For purposes of comparison it was desirable to know the properties of this glucoside and its tetraacetate, and since these data are not available in the literature it was necessary to synthesize these substances.

The work with gladiolus corms, which will be published in detail in a later paper, has shown, however, that the glycoside formed from *o*-chlorophenol is not  $\beta$ -*o*-chlorophenol-*d*-glucoside since acetylation of partially purified preparations of the glycoside has yielded a crystalline derivative which melts about 57° C. higher than synthetic  $\beta$ -*o*-chlorophenol-*d*-glucoside tetraacetate and has a considerably higher molecular weight as judged by the chlorine content. It was, therefore, thought desirable to publish separately the data on synthetic  $\beta$ -*o*-chlorophenol-*d*-glucoside.

### EXPERIMENTAL

*Synthesis of  $\beta$ -o-chlorophenol-d-glucoside tetraacetate.* The procedure used was based on the work of Helferich and Schmitz-Hillebrecht (5) who have prepared various phenol glycosides by direct reaction between the acetylated sugar and phenol in the presence of a suitable catalyst. A mixture of 128 g. (1 mole) of *o*-chlorophenol, 97 g. (0.25 mole) of pentaacetyl glucose, and 1.3 g. of *p*-toluenesulphonic acid was heated, with constant stirring, in an oil bath for 30 minutes at 115° C. followed by 20 minutes at 125°. After cooling, the material was dissolved in 300 cc. benzene and shaken with dilute NaOH solution to remove the excess *o*-chlorophenol. This procedure for the removal of the *o*-chlorophenol did not prove feasible be-

<sup>1</sup> Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 204. Copyright, 1940, by Boyce Thompson Institute for Plant Research, Inc.

cause of the formation of emulsions and the phenol was finally removed by distillation under vacuum (temperature less than  $30^{\circ}$ ) with repeated addition of water as required. The product crystallized on the addition of absolute alcohol to the contents of the flask after the *o*-chlorophenol had been driven off. After several recrystallizations from a mixture of absolute alcohol and acetone, 24 g., melting at  $150.5^{\circ}$  to  $151^{\circ}$  (Corr.), were obtained. This represents a yield of about 20 per cent based on the pentaacetyl glucose. Optical rotation was found to be  $[\alpha]_D^{23} = -44.6^{\circ}$  in  $\text{CHCl}_3$  (concn. 3.755 g.).

Analyses:<sup>2</sup> Calculated for  $\text{C}_{20}\text{H}_{23}\text{O}_{10}\text{Cl}$ : C, 52.35; H, 5.05; Cl, 7.73. Found: C, 52.49, 52.42; H, 5.36, 5.32; Cl, 7.58, 7.54.

*Deacetylation of  $\beta$ -o-chlorophenol-d-glucoside tetraacetate to form  $\beta$ -o-chlorophenol-d-glucoside.* A suspension of 7.46 g. of the tetraacetate in anhydrous methyl alcohol was cooled in an ice-salt mixture and the equivalent of 5 cc. 0.5 N barium methylate in methyl alcohol added (6, p. 1185). After standing in the icebox for about 24 hours 5 cc. of 0.5 N  $\text{H}_2\text{SO}_4$  were added and the solution filtered. On evaporation of the filtrate under vacuum the glucoside separated out in crystalline form. Three crops of crystals with a total weight of 4.21 g. (yield, 89 per cent) were obtained. The pure substance, recrystallized several times from ethyl acetate, melts at  $171^{\circ}$  to  $171.5^{\circ}$ , and has a specific rotation of  $[\alpha]_D^{26} = -65.3^{\circ}$  in absolute alcohol (concn. 3.57 g.). It is quite soluble in water and ethyl alcohol, less soluble in cold ethyl acetate.

Analyses:<sup>2</sup> Calculated for  $\text{C}_{12}\text{H}_{15}\text{O}_6\text{Cl}$ : C, 49.58; H, 5.20; Cl, 12.20. Found: C, 49.49, 49.67; H, 5.09, 5.26; Cl, 11.71, 11.70.

*Preparation of  $\beta$ -o-chlorophenol-d-glucoside tetraacetate by acetylation of the synthetic glucoside.* A 218 mg. portion of  $\beta$ -o-chlorophenol-d-glucoside was acetylated by dissolving in 10 cc. dry pyridine, adding 5 cc. of acetic anhydride, and allowing the reaction to proceed at room temperature overnight. The next morning the mixture was slowly poured, with constant stirring, into 75 cc. of ice-water. After standing for one-half hour the crystalline product was filtered off, washed with water, and dried in a  $\text{CaCl}_2$  desiccator. Yield, 336 mg., melting point,  $150.5^{\circ}$  to  $151^{\circ}$ . On recrystallization from absolute alcohol the melting point remained unchanged, and no depression of melting point resulted when mixed with some of the tetraacetate prepared in the original synthesis. Optical rotation was found to be  $[\alpha]_D^{24} = -44.2^{\circ}$  in  $\text{CHCl}_3$  (concn. 3.165 g.).

*Hydrolysis of  $\beta$ -o-chlorophenol-d-glucoside by emulsin.* To 32.8 mg. of  $\beta$ -o-chlorophenol-d-glucoside dissolved in 100 cc. of water were added 100 cc. of 0.1 M acetate buffer of pH 4.75 and 100 mg. of emulsin (British Drug Houses Ltd.). After 22 hours at  $35^{\circ}$  the mixture was distilled and 100 cc. of

<sup>2</sup> The author is indebted to Miss H. Jeanne Thompson for performing the microanalyses.



distillate collected. The *o*-chlorophenol in the distillate was determined by adding an excess of bromine in the form of a 0.1 N potassium bromate solution and determining the excess bromine iodometrically under the conditions recommended for the quantitative determination of phenol (1, p. 281). (Previous trials with this method had shown this to be a satisfactory quantitative method for the determination of *o*-chlorophenol, a dibromo derivative being formed.) The quantity of *o*-chlorophenol recovered in the distillate showed that 93 per cent of the glucoside present had been hydrolyzed. As a control, 26.3 mg. of glucoside were carried through the same procedure except that the emulsin was omitted. The distillate from this control sample absorbed only a trace of bromine.

#### SUMMARY

$\beta$ -*o*-chlorophenol-*d*-glucoside tetraacetate was prepared by heating *o*-chlorophenol with pentaacetyl glucose in the presence of *p*-toluenesulphonic acid. On deacetylation crystalline  $\beta$ -*o*-chlorophenol-*d*-glucoside was obtained.

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# CONTROL OF BUD GROWTH AND INITIATION OF ROOTS AT THE CUT SURFACE OF POTATO TUBERS WITH GROWTH-REGULATING SUBSTANCES<sup>1</sup>

JOHN D. GUTHRIE

The great interest shown recently in growth-regulating substances of the auxin type raised the question of the relation of these substances to the rest period or dormancy shown by certain plants. Furthermore, a number of papers have appeared in which a shortening of the rest period following treatment with synthetic growth-regulating substances has been reported. On the contrary, the inhibiting action of these substances on bud growth observed originally by Thimann and Skoog (14) offered a possible explanation for the dormancy of buds during the rest period. It has been shown by Denny (4) that another group of chemical substances of which ethylene chlorohydrin, potassium thiocyanate, and thiourea are examples, is very effective in breaking the rest period of plants by stimulating prompt growth of the buds. An obvious question is whether these dormancy-breaking chemicals act by either increasing or decreasing the auxin content of the tissue. An analytical answer to this question is difficult at present due to the inadequacy of methods for quantitatively extracting auxin from tissues. Some data using available methods will be presented.

Although most of the experiments reported in this paper are open to the criticism that the synthetic growth substances used most frequently in experimental work are not natural auxins and may act differently from natural auxins, little support is offered for the idea that the rest period is controlled by changes in the amount or activity of auxin-like substances in the tissues. It will be obvious to the reader, however, that certain of the results presented in this paper, considered separately, might be regarded as supporting such an idea. Of especial interest in this respect is the fact that potato tubers may be put into a state resembling natural dormancy by treatment with naphthaleneacetic acid or its methyl ester and then made to grow again by treatment with ethylene chlorohydrin (9, 10). However, it is likely that if some inhibiting substance does explain the dormancy of buds, it is different from auxin.

Certain of the experiments have a bearing on the converse question of what effect the state of dormancy has on the action of synthetic growth-regulating substances, since it was observed that old or non-dormant tubers rooted more readily at the cut surface than dormant or freshly-harvested tubers following treatment with indoleacetic acid and, furthermore,

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rooting at the cut surface induced by indoleacetic acid was increased by a preliminary treatment with ethylene chlorohydrin.

INHIBITION OF THE SPROUTING OF NON-DORMANT POTATO TUBERS  
BY TREATMENT WITH INDOLEACETIC ACID OR  
NAPHTHALENEACETIC ACID

As shown in previous publications (7, 8, 9), indoleacetic acid and naphthaleneacetic acid have a marked inhibiting action on the sprouting of old or non-dormant potato tubers. Additional experiments have been made to establish certain details of concentration and duration of treatment.

*Method of treatment.* Most of the treatments were made by the basal soak method. Approximately cubical pieces with one non-sprouting eye in the center of one side were cut from potato tubers (*Solanum tuberosum* L.). These pieces were washed and dried with cheesecloth. They were then mixed and arranged in open Petri dishes, 12 to a dish, with the eyes up. After the dishes had been placed in a room at 10° C., the solution was poured into the dishes; usually 125 cc. were used, which came about half-way up the sides of the pieces. After a stated interval, the pieces were planted in soil, 12 to a flat. The flats were usually piled up in the basement at about 25° C. for observation. In some cases the flats were placed in the greenhouse or outside, depending on the season. The flats were observed at intervals of three to seven days and the number of pieces showing a sprout above ground recorded. From these data the time in days after planting for half of the pieces to show sprouts above ground was obtained. This is essentially the time for the median piece to sprout.

*Indoleacetic acid.* The effect of various concentrations of neutralized 3-indoleacetic acid on the sprouting of non-dormant potato tubers is shown in Table I. The details of the experiments are as follows:

Exp. 1. Irish Cobbler tubers from New Jersey, harvested about three months previously, were used. The pieces weighed about 10 g. each. After three days a thin slice was cut from the bottom of each piece, a fresh solution added, and the treatment continued for three more days. The indoleacetic acid was neutralized with an equivalent amount of sodium hydroxide.

Exp. 2. Irish Cobbler tubers from Connecticut, harvested about five months previously, were used. The procedure was the same as in Exp. 1, except that the potassium salt of indoleacetic acid was used.

Exp. 3. Bliss Triumph tubers from Florida which had probably been in storage several months were used. The pieces weighed about 20 g. The potassium salt was used.

Exp. 4. Old Green Mountain tubers bought in the local market were used. Pieces weighing 10 g. were treated for intervals of 3 days, 18 hours, and 4 hours with the potassium salt.

Exp. 5. Old Green Mountain tubers bought in the local market were used. Pieces weighing 10 g. were treated with the potassium salt.



The results, summarized in Table I, show that potato tubers which sprout 50 per cent in two weeks or less, require more than three weeks to show the same per cent sprouting following treatment with a solution containing 250 mg. per liter for three days or longer. Higher concentrations bring about a more marked inhibition of sprouting. For example, in Exp. 3 all of the 12 pieces were in sound condition 100 days after treatment with the potassium salt of indoleacetic acid, 1000 mg. per liter for three days. Only one piece had sprouted. After 156 days only four of the pieces had sprouted and the rest rotted without sprouting. A treatment with 500 mg. per liter for three days was included in Exp. 3 but is not given in the table. Half of the pieces were above ground in 118 days, and nine pieces sprouted after 198 days. Short intervals of treatment inhibited sprouting if a high concentration was used. For example, Exp. 5 shows that pieces which show 50 per cent sprouts above ground in nine days require 25 days to show the same per cent sprouting following a two-hour treatment with a solution containing 1000 mg. of the potassium salt of indoleacetic acid per liter.

TABLE I  
INHIBITION OF THE SPROUTING OF NON-DORMANT POTATO TUBERS BY TREATMENT WITH  
NEUTRALIZED INDOLEACETIC ACID

Conc., mg. per liter	Days for 50 per cent above ground									
	Exp. 1	Exp. 2	Exp. 3	Exp. 4			Exp. 5			
	Treat- ed 6 days	Treat- ed 6 days	Treat- ed 3 days	Treat- ed 3 days	Treat- ed 18 hours	Treat- ed 4 hours	Treat- ed 16 hours	Treat- ed 8 hours	Treat- ed 4 hours	Treat- ed 2 hours
1000	>66*	>66	>156				28	25	25	25
250	33*	30	31	20	13	13				
100	18*	14								
0 (H <sub>2</sub> O)	16	11	9	10	10	10	9	9	9	9

\* Free acid neutralized with equivalent amount of NaOH. In all other experiments the potassium salt of indoleacetic acid was used.

Rooting at the cut surface such as has been reported previously (8) was observed in most of the treated lots. Experiments dealing specifically with such rooting will be described in a later section of this paper. It is well known that synthetic growth-regulating substances hasten the rooting of cuttings—see Zimmerman and Hitchcock (19).

*Naphthaleneacetic acid.* The inhibiting effect of the potassium salt of  $\alpha$ -naphthaleneacetic acid on the sprouting of potato tubers is shown in Table II. Green Mountain tubers from the local market were used in Exps. 1 and 2. In Exp. 3 Bliss Triumph tubers from Florida bought locally were used. In all three experiments pieces weighing 20 g. were used.

The results show that a solution of 20 mg. per liter brings about considerable inhibition of sprouting when applied for one day and marked in-

hibition if applied for six days. Use of higher concentrations results in marked inhibition even if applied for short intervals. For example, treatment with a solution of 200 mg. per liter for two hours inhibited sprouting for about 100 days. In an experiment which is not included in the table, the pieces were treated by completely covering them with the solution for two hours at 22° C., instead of using the basal soak procedure at 10° C. Marked inhibition of sprouting was observed with 25 mg. per liter, while with 200 mg. per liter only two pieces sprouted, although the rest of the pieces remained in good condition for 100 days.

TABLE II  
INHIBITION OF THE SPROUTING OF NON-DORMANT POTATO TUBERS BY TREATMENT WITH THE POTASSIUM SALT OF NAPHTHALENEACETIC ACID

Conc., mg. per liter	Days 50 per cent above ground			
	Exp. 1			
	Treated 1 day	Treated 2 days	Treated 3 days	Treated 6 days
100	> 130	> 120	127	> 124
20	30	38	43	71
0 (H <sub>2</sub> O)	7	8	9	8
	Exp. 2			
	Treated 2 hours	Treated 4 hours	Treated 16 hours	
	100	125	> 125	
0 (H <sub>2</sub> O)	8	8	8	
	Exp. 3			
	Treated 1 day	Treated 2 days	Treated 3 days	
	80	> 88	35	> 88
40	72	54	30	
20	32	30	38	
10	28	26	27	
5	26	24	26	
0 (H <sub>2</sub> O)	24	24	24	

A comparison of Table II with Table I shows that naphthaleneacetic acid is about ten times as effective as indoleacetic acid in inhibiting the sprouting of potato tubers. Rooting was observed at the cut surface in all of the treated lots, but it started much later and fewer pieces showed roots than observed in the experiments with indoleacetic acid.

EFFECT OF CHEMICALS THAT BREAK THE REST PERIOD ON THE  
SPROUTING OF POTATO TUBERS MADE "DORMANT" BY  
TREATMENT WITH NAPHTHALENEACETIC ACID

Since treatment with indoleacetic acid and naphthaleneacetic acid in-

duced a condition in potato tubers resembling natural dormancy inasmuch as the tubers so treated did not grow when planted, it was a logical step to see if dormancy-breaking chemicals such as ethylene chlorohydrin and potassium thiocyanate would break this "dormancy." Tubers were first treated with the potassium salt of indoleacetic acid and then with either ethylene chlorohydrin or potassium thiocyanate. Only slight increases in the rate of sprouting were observed. Therefore attention was shifted to tubers whose sprouting had been inhibited with naphthaleneacetic acid. This substance is much more effective than indoleacetic acid in inhibiting bud growth and producing marked inhibition at low concentrations. Pieces of tubers, the sprouting of which was inhibited by treatment with naphthaleneacetic acid, sprouted promptly when treated with ethylene chlorohydrin. Potassium thiocyanate, however, had little effect. This is shown

TABLE III

EFFECT OF CHEMICALS THAT BREAK THE REST PERIOD ON THE SPROUTING OF POTATO TUBERS MADE "DORMANT" BY TREATMENT WITH THE POTASSIUM SALT OF NAPHTHALENEACETIC ACID

Pieces treated with potassium salt of naphthaleneacetic acid and then treated as follows:	Days for 50 per cent above ground*		
	Exp. 1	Exp. 2	Exp. 3
Ethylene chlorohydrin dip 25 cc. of 40 per cent per liter	10, 10	10, 10	17, 17
Control—H <sub>2</sub> O	43, 43	27, 27	> 134, > 134
Ethylene chlorohydrin vapor 1.5 cc. of 40 per cent per 700 g.	13, 12	10, 13	
Control—closed container	48, 53	32, 35	
KSCN, 20 g. per liter, 2 hour soak	18, 20	28, 27	
KSCN, 10 g. per liter, 2 hour soak	48, 24		
Control—H <sub>2</sub> O	48, 27	27, 32	

\* Values separated by commas are duplicates.

in Table III. The details of the experiments are as follows:

Exp. 1. Green Mountain tubers from the local market were used. Untreated pieces from these showed 50 per cent above ground in nine days. Pieces weighing about 20 g. were cut from these and treated by the basal soak method for four days at 10° C. with the potassium salt of naphthaleneacetic acid, 100 mg. per liter. The pieces were planted for four days. They were then dug up, washed, and, except in the case of the chlorohydrin vapor treatment, the callus tissue cut off in a thin layer. The pieces were then mixed and treated with ethylene chlorohydrin and potassium thiocyanate as shown in Table III. The ethylene chlorohydrin treatments were for 24 hours.

Exp. 2. Another lot of Green Mountain tubers was used in this experiment. Untreated pieces showed 50 per cent sprouting 12 days after planting. The treatments were the same as in Exp. 1, except that the preliminary

treatment with the potassium salt of naphthaleneacetic acid was for only three days, and the treatments with ethylene chlorohydrin and potassium thiocyanate were made six days later. All the pieces had the callus tissue cut off prior to the second treatment.

Exp. 3. Bliss Triumph tubers from Florida, purchased in the local market were used for this experiment. Untreated tubers from this lot showed 50 per cent sprouting in 24 days. The preliminary treatment with the potassium salt of naphthaleneacetic acid was the same as in Exp. 1. Treatments by the ethylene chlorohydrin dip method were made after the tubers had been planted five days. Callus tissue was cut off. The pieces and plants were dug up and photographed 50 days after the start of the experiment as shown in Figure 1.

Table III and Figure 1 show clearly that pieces of non-dormant potato tubers which sprout promptly when planted may have their sprouting retarded or prevented by treatment with the potassium salt of naphthaleneacetic acid. If such pieces are then treated with ethylene chlorohydrin, they again grow promptly. Thus naphthaleneacetic acid induces a condition in the tubers resembling natural dormancy inasmuch as it can be broken with ethylene chlorohydrin. A preliminary report has been made of this (9). However, Table III shows that potassium thiocyanate which is very effective in breaking natural dormancy of potato tubers (4) has only slight effect on the "dormancy" induced by naphthaleneacetic acid. Although natural dormancy in potato tubers may be due to the presence of an inhibiting substance similar in action to naphthaleneacetic acid, these experiments show that such an inhibitor must be different in its behavior toward potassium thiocyanate.

#### INHIBITION OF THE SPROUTING OF THE BUDS OF POTATO TUBERS WITH THE VAPOR OF THE METHYL ESTER OF NAPHTHALENEACETIC ACID

All the experiments reported previously in this paper have been with cut pieces of tubers. From a practical standpoint it was desirable to inhibit the growth of the buds of whole tubers. It was found that this could be accomplished by spraying the tubers with naphthaleneacetic acid dissolved in a mixture of olive oil and acetone and storing at 10° C. or by inserting toothpicks soaked in a solution of the potassium salt into the tubers, a procedure similar to that used by Schneider (12). However, these methods have certain obvious objections from a practical standpoint.

The best method for treating whole tubers is with a vapor. Since it seemed likely that the methyl ester of naphthaleneacetic acid would be slightly volatile, it was tried and found to be effective as previously reported (10). In order to determine the limits of concentration, temperature, and time for such treatments, additional experiments were made.



Various amounts of the methyl ester were dissolved in 5 cc. of acetone and poured on four 11 cm. filter papers. After the acetone had evaporated, the four papers were placed on top of six Bliss Triumph tubers in an

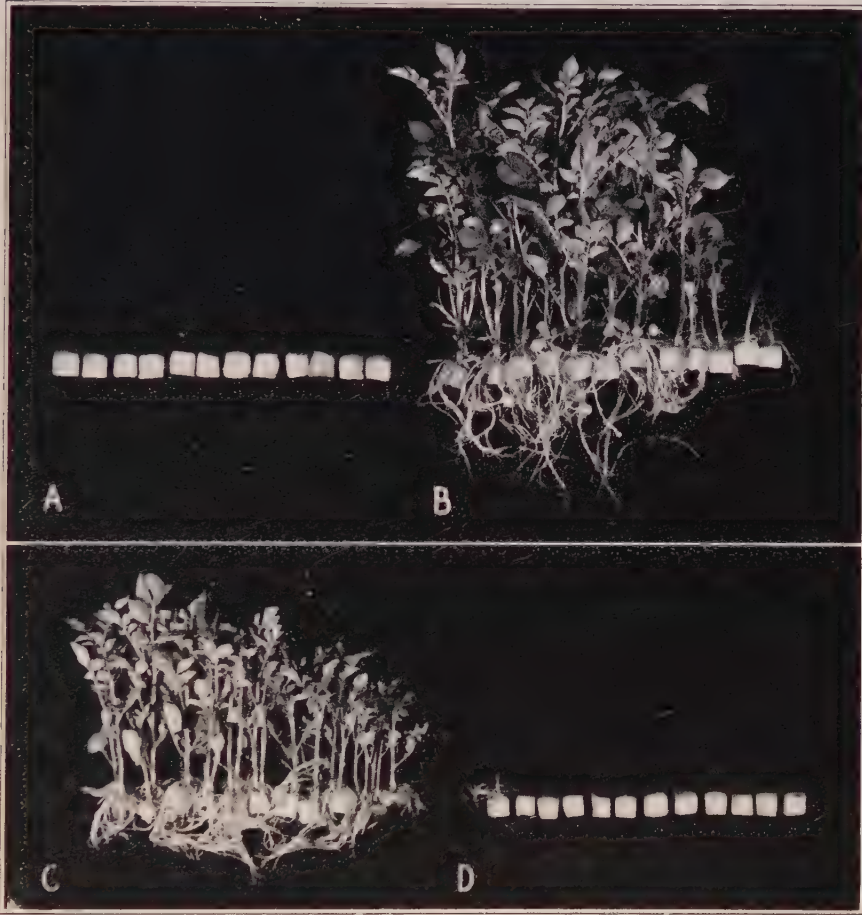


FIGURE 1. Inducing dormancy in potato tubers with the potassium salt of naphthaleneacetic acid and then breaking it with ethylene chlorohydrin. A. Pieces treated with the potassium salt of naphthaleneacetic acid, 100 mg. per liter for 4 days. B. Control pieces treated with water. C. Pieces like those shown in A treated with ethylene chlorohydrin, 24 hr. dip, 25 cc. of 40 per cent per liter. D. Pieces like those in A, treated with water.

enameled can and covered with half of a Petri dish but not sealed. One series was left in the laboratory at 26° to 30° C., another in a room at 22° C., and another in a room held at 10° C. Observations were made on each tuber at intervals, and the total number of buds or eyes on the tubers as

well as the number of buds sprouting was recorded. The results are given in Table IV, Exp. 1 A, where the totals for the six tubers in each lot are given. The experiment shows that at temperatures of 22° and above, considerable inhibition of bud growth was produced by as little as 2.5 mg. of the methyl ester per six tubers, and marked inhibition with 22 mg. or over. At 10° C. the inhibition was less marked, but clearly evident with 22 mg. or over. The volatility of the methyl ester of naphthaleneacetic acid at 10° C. may be demonstrated by the production of epinasty of the leaves of tomato

TABLE IV

INHIBITION OF THE SPROUTING OF THE BUDS OF POTATO TUBERS WITH THE VAPOR OF THE METHYL ESTER OF NAPHTHALENEACETIC ACID

Temp.	Mg. methyl ester of naphthaleneacetic acid	Exp. 1 A						Exp. 1 B			
		Total No. buds	No. buds sprouting per 6 tubers				Total No. eyes	No. buds sprouting per 6 tubers			
			After 22 days	After 36 days	After 51 days	After 86 days		After 46 days	After 63 days	After 90 days	
Room, 26°-30° C.	200	45	0	0	0	1	42	0	0	0	
	65	47	0	0	0	4	41	0	0	0	
	22	39	0	0	1	14	38	0	0	4	
	7.5	44	0	0	10	18	38	1	8	16	
	2.5	47	0	9	24	33	52	0	37	42	
	0	46	11	21	24	32	39	0	10	11	
22° C.	200	40	0	0	0	2	44	0	0	0	
	65	47	0	0	0	2	44	0	0	0	
	22	49	0	1	1	12	46	0	0	9	
	7.5	49	0	4	14	24	41	1	6	10	
	2.5	48	0	13	31	32	46	2	13	31	
	0	42	15	23	23	23	42	2	9	16	
10° C.	200	48	0	0	0	6					
	65	39	0	0	0	10					
	22	49	0	0	0	16					
	7.5	51	0	0	0	38					
	2.5	49	0	0	7	42					
	0	48	0	0	1	43					

plants at this temperature. Production of epinasty at room temperature has been reported in a previous paper (10).

The papers impregnated with the methyl ester were removed from the room temperature and 22° C. lots 57 days after the start of Exp. 1 A and placed with Irish Cobbler tubers from South Carolina to form Exp. 1 B. These tubers were slightly dormant at the start of the experiment, but the control tubers broke dormancy and sprouted after 63 days. The results show that sufficient of the methyl ester remained on the papers impregnated originally with 22 mg. or more of the methyl ester to inhibit a second lot of tubers. This experiment shows that the methyl ester vaporizes very

slowly and is given off in small amounts over a long period. The papers that originally held 2.5 mg. of the ester appear to have slightly increased the sprouting of the second lot of tubers. A statistical examination of the values for individual tubers of these lots shows that there is a significant difference between the lots whose papers held originally 2.5 mg. of the ester and the controls. This indication that low concentrations of the ester may show a slight stimulating action should be investigated further.

In order to find how short a period of exposure to the vapor of the methyl ester of naphthaleneacetic acid might be effective, 30 partially dormant Irish Cobbler tubers from South Carolina were placed in a partly closed container with five 18.5 cm. filter papers impregnated with a total of 400 mg. of the ester and placed at 24° C. A similar lot of tubers was used as controls, using plain filter paper. Lots of six tubers were removed from these containers at intervals and the sprouting recorded. The results are given in Table V. It will be seen that exposure of even one day to the vapor

TABLE V  
SPROUTING OF POTATO TUBERS EXPOSED TO THE VAPOR OF THE METHYL ESTER OF  
NAPHTHALENEACETIC ACID FOR VARIOUS PERIODS

Treatment	Time of exposure	Total No. eyes	No. eyes sprouting		
			After 46 days	After 56 days	After 83 days
Methyl ester of naphthaleneacetic acid, vapor	1 day	45	0	0	9
	3 days	47	0	0	7
	7 days	52	0	0	0
	14 days	39	0	0	1
	Continuous	41	0	0	0
Control, paper and container only	1 day	47	3	14	22
	3 days	46	9	18	20
	7 days	42	6	11	23
	14 days	41	6	25	18
	Continuous	42	0	12	40

of the methyl ester of naphthaleneacetic acid produces considerable inhibition of the growth of the buds and marked inhibition was obtained with exposures of seven days or more. These results suggest that if treatment with the methyl ester of naphthaleneacetic acid should be coupled with storage at reduced temperature, it might be best to expose the tubers to the vapor of the methyl ester for a week at about 24° C. and then transfer them to a lower temperature.

The buds of the tubers treated with the vapor of the methyl ester of naphthaleneacetic acid can be made to grow again by treatment with the vapor of ethylene chlorohydrin. In one such experiment, tubers, the sprouting of which had been inhibited by treatment with the vapor of the methyl ester, were divided into two lots and one was treated one month

later with the vapor of ethylene chlorohydrin. The tubers were photographed after three more weeks as shown in Figure 2 A, B, C. It will be



FIGURE 2. Inhibiting effect of the vapor of the methyl ester of naphthaleneacetic acid on the sprouting of potato tubers. A. Tubers treated with the vapor of the methyl ester of naphthaleneacetic acid and later with ethylene chlorohydrin. B. Tubers treated only with the methyl ester. C. Control tubers, untreated. D. Tubers treated with the vapor of the methyl ester of naphthaleneacetic acid photographed after 108 days. E. Control tubers photographed after 108 days.

seen that the tubers treated with ethylene chlorohydrin after treatment with the methyl ester sprouted just as well as the control tubers that had



no treatment, while only a few sprouts appeared on the tubers treated with the methyl ester alone.

Exposure to the vapor of the methyl ester of naphthaleneacetic acid not only inhibits the sprouting, but retards the withering of the tubers. This is shown in Figure 2 D, E. It will be noted that the treated tubers are still rather plump and sproutless after 108 days, while the control tubers have many large sprouts and are almost withered away. This result has been observed consistently in other experiments.

In some miscellaneous experiments it was noted that treatment of six tubers with 0.8 cc. of acetonitrile for 24 hours in a closed container retarded sprouting about 30 days. Since this compound has been shown by Wächter (17) to produce epinasty of leaves and ethylene which also produces epinasty of leaves as shown by Crocker, Zimmerman, and Hitchcock (3) retards the growth of potato buds as first indicated by the experiments of Elmer (6), it would appear that the capacity to retard bud growth is a property frequently possessed by substances that produce epinasty. The vapor of the ethyl ester of naphthaleneacetic acid also produces marked inhibition of the growth of the buds of potato tubers and appears to be approximately as effective as the methyl ester.

#### INITIATION OF ROOTS AT THE CUT SURFACE OF POTATO TUBERS BY TREATMENT WITH INDOLEACETIC ACID AND NAPHTHALENEACETIC ACID

As reported previously (8) treatment of potato tubers by the basal soak method with solutions of the potassium salt of indoleacetic acid induced roots to grow on the cut surface of the pieces. This seemed to be of some interest, since there appears to be no previous record of roots growing from the cut surface of potato tubers, and it appeared that this was a case in which rooting was induced at a place where it never occurred without treatment. This, however, is not the case, since one case of cut surface rooting was observed on an untreated piece out of some 500 untreated pieces carefully observed for long periods during the course of the following experiments.

The method of treatment for the study of the rooting at the cut surface was essentially the same basal soak described previously in this paper. Unless otherwise stated the temperature was 10° C. In order to avoid complications due to the sprouting of the buds, the pieces were cut without an eye or bud and consequently they did not sprout. The pieces were approximately cubical and in some cases the pieces were cut from the center of the tuber, avoiding the skin and cortex ring as much as possible.

*Indoleacetic acid.* The effect of the potassium salt of indoleacetic acid on the rooting at the cut surface was studied in several experiments, the details of which are as follows:

Exp. 1. Bliss Triumph tubers from Florida bought in the local market were used.

Exps. 2, 3, 4. Green Mountain tubers were used.

Exp. 5. Green Mountain tubers were used. The pieces were small, weighing only about 3 g.

Exp. 6. The same tubers were used as in Exp. 4 but pieces about  $3 \times 5 \times 15$  mm. were cut with skin at the end. They weighed about 0.5 g. and were treated by placing on filter papers wet with the solution in closed Petri dishes for one and two days. Each lot consisted of 24 pieces instead of the usual 12 pieces.

Some of the results are given in Table VI. Although observations of the rooting of the pieces were made at frequent intervals, for the sake of

TABLE VI  
EFFECT OF THE POTASSIUM SALT OF INDOLEACETIC ACID ON ROOTING AT THE CUT SURFACE OF POTATO TUBERS

Conc., mg. per liter	Number of pieces rooting at cut surface							
	Exp. 1, 20 g. pieces treated 3 days				Exp. 2, 10 g. pieces treated 4 days			
	With skin		Without skin		With skin		Without skin	
	After 23 days	Total	After 23 days	Total	After 12 days	Total	After 12 days	Total
1000	4	8	0	3	7	12	0	3
500	4	5	0	2				
250	0	2	0	1				
0 (H <sub>2</sub> O)	0	0	0	0	0	0	0	0
Exp. 3, 10 g. pieces								
Treated 1 day				Treated 3 days				
With skin		Without skin		With skin		Without skin		
After 24 days	Total	After 24 days	Total	After 24 days	Total	After 24 days	Total	Total
250	2	3	0	1	5	10	0	8
0 (H <sub>2</sub> O)	0	0	0	0	0	0	0	0

brevity only the number rooting shortly after rooting began and the total number of pieces rooting are given. It will be noted in Table VI that the pieces cut with skin, which contain more vascular tissue, rooted sooner and more completely than pieces cut without skin from the center of the tubers, which contain less vascular tissue. The best rooting appears to be obtained with 1000 mg. per liter at 10° C. for a period of three or four days. Since it was thought that a temperature of 10° C. might not always be available to those desiring to induce rooting at the cut surface of potato tubers, 27° C. was compared with 10° C. in Exp. 4, the results of which are

shown in Table VII. Short intervals were used since experience had shown that the pieces were injured by long treatments at high temperatures. The pieces were with skin and weighed 10 g. The data show that good rooting could be obtained when the treatments were made at 27° for two hours at a concentration of 1000 mg. per liter. The treatment at 10° C. also pro-

TABLE VII

ROOTING AT THE CUT SURFACE OF POTATO TUBERS INDUCED BY TREATMENT WITH THE POTASSIUM SALT OF INDOLEACETIC ACID AT DIFFERENT TEMPERATURES

Conc., mg. per liter	Number of pieces rooting at cut surface							
	Treated 2 hours				Treated 4 hours			
	10° C.		27° C.		10° C.		27° C.	
	After 11 days	Total	After 11 days	Total	After 11 days	Total	After 11 days	Total
1000 0 (H <sub>2</sub> O)	2 0	9 0	5 0	11 0	5 0	12 0	5 0	10 0

duced good rooting when applied for two hours but somewhat better rooting when applied for four hours. Exp. 5, the results of which are not included in the tables, showed that rooting could be obtained with 3 g. pieces with skin treated with 500 mg. per liter for four days at 10° C., and with 3 g. pieces without skin at a concentration of 250 mg. per liter. Exp. 6, also not reported in the tables, showed that rooting at the cut surface of pieces as small as 0.5 g. could be obtained at a concentration of 1000 mg. per liter for two days at 10° C.

The rooting at the cut surface is further illustrated by Figure 3. The 12 rooting pieces on the left were treated six weeks previously for three days with a solution of 1200 mg. of the potassium salt of indoleacetic acid per liter. The 12 non-rooting pieces on the right were water controls. Figure 4 shows an example of cut surface rooting from a piece cut from the center of a tuber without skin or buds. This is a piece from Exp. 3, treated three days at 10° C. by the basal soak method with a solution of the potassium salt of indoleacetic acid, 250 mg. per liter.

Since the pieces used in these rooting experiments had no buds, they did not sprout, but eventually rotted away. Most of the pieces were in good condition after 100 days. One or two pieces often remained after 200 days. The pieces cut without skin rotted sooner than those with skin.

*Naphthaleneacetic acid.* Experiments similar to those with indoleacetic acid were made with naphthaleneacetic acid, using the potassium salt. Old Green Mountain tubers were used in Exps. 1, 2, and 3. The pieces weighed 20 g. Only the observations for pieces cut without skin are shown in Table VIII. Pieces without skin were included in Exp. 1 but poorer rooting was

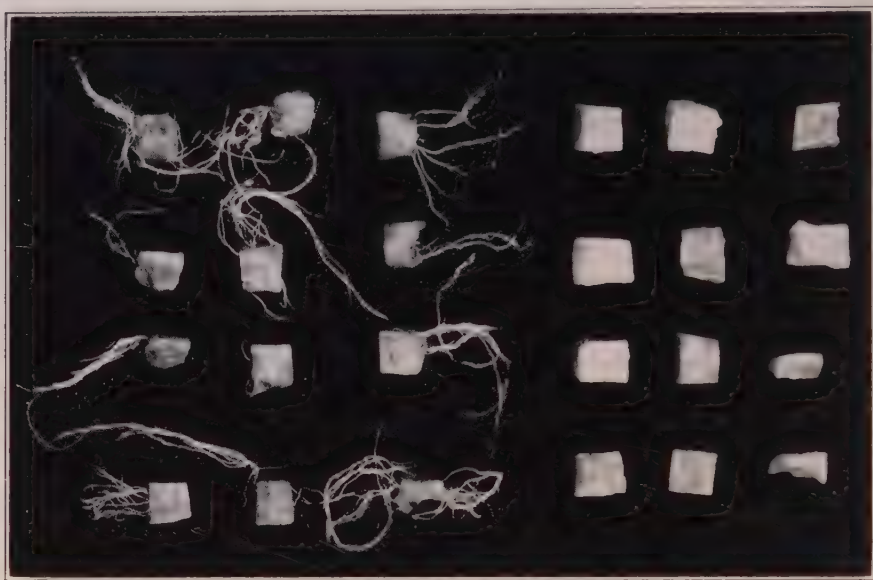


FIGURE 3. Induction of roots on the cut surface of potato tubers. Pieces on the left were treated with a solution of the potassium salt of indoleacetic acid, 1200 mg. per liter for 3 days at  $10^{\circ}$  C.; pieces on the right were water controls.

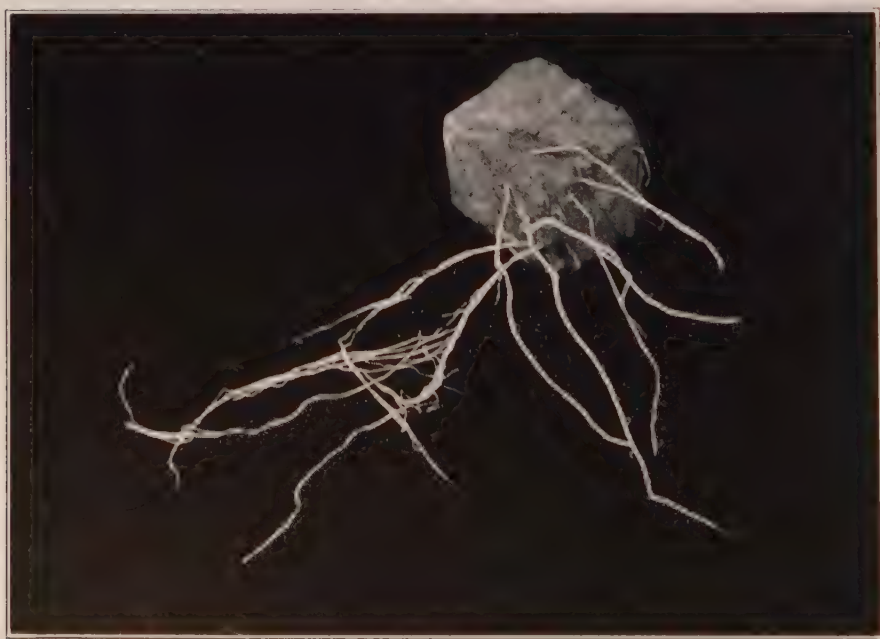


FIGURE 4. Rooting at the cut surface of a piece cut from the center of a tuber, without skin or buds. This piece was treated for 3 days at  $10^{\circ}$  C. with a solution of 250 mg. of the potassium salt of indoleacetic acid per liter.



obtained. Higher concentrations than those given in Table VIII were tried in preliminary experiments, but tended to produce injury. The results show that the potassium salt of naphthaleneacetic acid induces rooting at the cut surface of potato tubers at less than one-tenth the concentration

TABLE VIII

EFFECT OF THE POTASSIUM SALT OF NAPHTHALENEACETIC ACID ON ROOTING AT THE CUT SURFACE OF POTATO TUBERS

Number of pieces rooting at cut surface												
Conc., mg. per liter	Exp. 1											
	Treated 1 day			Treated 2 days			Treated 3 days			Treated 6 days		
	After 45 days	After 60 days	Total	After 45 days	After 60 days	Total	After 44 days	After 59 days	Total	After 44 days	After 59 days	Total
100	0	1	8	0	2	8	1	3	11	0	1	4
20	1	6	12	0	5	11	2	4	9	3	3	8
0 (H <sub>2</sub> O)	0	0	0	0	0	0	0	0	0	0	0	1

Exp. 2										
	Treated 2 hours		Treated 4 hours		Treated 16 hours		Treated 24 hours		Treated 48 hours	
	After 56 days	Total	After 56 days	Total	After 56 days	Total	After 56 days	Total	After 56 days	Total
200	0	2	4	8	6	7	3	5	3	10
0 (H <sub>2</sub> O)			0	0	0	0			0	0

Exp. 3									
	Treated 1 day			Treated 2 days			Treated 3 days		
	After 41 days	After 55 days	Total	After 41 days	After 55 days	Total	After 41 days	After 55 days	Total
80	1	6	6	2	7	8	0	0	3
40	3	7	9	2	9	11	3	4	5
20	2	5	9	4	6	10	3	5	8
10	1	5	5	4	6	8	2	6	8
5	1	2	3	3	5	6	3	6	9
0 (H <sub>2</sub> O)	0	0	0	0	0	0	0	0	0

required for the potassium salt of indoleacetic acid. It appears to work best at 10 to 40 mg. per liter for one to three days. Shorter treatments with 200 mg. per liter also produced good rooting. However, it will be noted that the rooting started much more slowly than with indoleacetic acid. Tables VI and VII show that rooting with the indole compound began in 11 to 24 days, while Table VIII shows that rooting with the naphthalene compound began about 40 days after treatment. The more rapid appearance of roots

with indoleacetic acid makes this more satisfactory for demonstration of rooting at the cut surface of potato tubers.

RELATION OF ROOTING AT THE CUT SURFACE TO THE STAGE  
OF THE REST PERIOD OR DORMANCY OF THE TUBERS

In some experiments to be described later in this paper, freshly-harvested or dormant potato tubers were treated with the potassium salt of indoleacetic acid. It was observed that the rooting at the cut surface was much less frequent than with old or non-dormant tubers used in other experiments. Therefore, the effect of the potassium salt of indoleacetic acid on the rooting of potato tubers in different stages of the rest period was investigated. The results are shown in Table IX. In Exps. 1 and 2 the tubers

TABLE IX

INFLUENCE OF TIME AFTER HARVEST ON THE ROOTING AT THE CUT SURFACE OF IRISH  
COBBLER POTATO TUBERS FOLLOWING TREATMENT WITH THE POTASSIUM SALT OF  
INDOLEACETIC ACID

Exp. No.	Source of tubers	Time since harvest	No. rooting at cut surface				
			After 14 days	After 28 days	After 60 days	After 86 days	After 182 days
1	New Jersey	0.5 months	0, 0	0, 1	0, 1	0, 2	8, 7
	South Carolina	7.0 months	6, 5	12, 12	12, 12		
	Institute gardens	3.5 months	1, 0	12, 11	12, 11		
2	New Jersey	1.3 months	0, 0	2, 0	6, 1	9, 6	
	South Carolina	8.0 months	0, 2	12, 12	12, 12		
3	New Jersey	2.0 months	0	3	12		

from New Jersey, harvested only 0.5 to 1.3 months before treatment, rooted much more slowly and less completely than tubers of the same variety that had been in storage from 3.5 to 8 months. The same lot of tubers from New Jersey also rooted more readily and completely as the period of storage increased as shown in Exps. 1, 2, and 3.

The above results, which show clearly that non-dormant tubers root much more readily than dormant tubers when treated with the potassium salt of indoleacetic acid, suggested that a preliminary treatment of dormant potato tubers with ethylene chlorohydrin might make them root more readily. Experiments reported in Table X show this to be the case. Freshly-harvested Irish Cobbler potato tubers were used in all four experiments. The ethylene chlorohydrin vapor treatments were made by the method of Denny (4), 3 cc. of 40 per cent ethylene chlorohydrin per kg. for 24 hours being used, with the exception of the second treatment in Exp. 4 where 1.5 cc. were used. In all cases one-eye pieces were planted to show that the treatment had broken the dormancy. The ethylene chlorohydrin-treated

lots showed 50 per cent sprouting in 17 to 23 days, while the untreated lots required 40 to 59 days. At stated intervals after the ethylene chlorohydrin treatment approximately 20 g. pieces with skin, but no eye, were cut from the tubers and both treated and control lots were treated in duplicate groups of 12 pieces with indoleacetic acid, 500 mg. per liter, neutralized

TABLE X

EFFECT OF BREAKING THE DORMANCY WITH A PRELIMINARY ETHYLENE CHLOROHYDRIN TREATMENT ON THE ROOTING AT THE CUT SURFACE OF POTATO TUBERS FOLLOWING A SUBSEQUENT TREATMENT WITH NEUTRALIZED INDOLEACETIC ACID

Exp. No.	Preliminary treatment	Treated with Na indoleacetate after	Number rooting at cut surface				
			After 3 weeks	After 5 weeks	After 7 weeks	After 10 weeks	After 12 weeks
1	Ethylene chlorohydrin	7 days	6, 5	12, 12	12, 12	12, 12	12, 12
	Control	7 days	1, 2	5, 7	9, 11	10, 12	11, 12
2	Ethylene chlorohydrin	5 days	0, 0	2, 3	6, 6	11, 12	11, 12
	Control	5 days	0, 0	1, 0	3, 0	10, 6	12, 12
	Ethylene chlorohydrin	9 days		3, 5	6, 8	10, 11	11, 12
	Control	9 days		0, 0	2, 0	5, 5	12, 12
3	Ethylene chlorohydrin	2 days	4, 3	8, 6	9, 9	10, 11	11, 12
	Control	2 days	0, 0	3, 2	3, 3	3, 3	3, 4
	Ethylene chlorohydrin	6 days	0, 0	5, 5	6, 10	7, 10	9, 11
	Control	6 days	1, 0	1, 3	3, 4	3, 5	5, 7
4	Ethylene chlorohydrin	10 days	0, 0	2, 3	10, 9	11, 9	12, 11
	Control	10 days	0, 0	2, 1	5, 2	5, 3	6, 8
	Ethylene chlorohydrin	4 days	0, 0	5, 3	9, 5	10, 6	12, 10
	Ethylene chlorohydrin	4 days	0, 0	8, 5	9, 6	12, 8	12, 11
	Control	4 days	0, 0	1, 3	3, 3	4, 4	5, 5
	Ethylene chlorohydrin	8 days	0, 0	0, 1	2, 4	4, 4	9, 8
	Ethylene chlorohydrin	8 days	0, 0	0, 1	4, 5	5, 8	6, 8
	Control	8 days	0, 0	1, 2	3, 5	4, 6	9, 7

with sodium hydroxide. The potassium salt was no longer available. The basal soak method for three days at 10° C. was used. The results show that with the exception of the eight-day part of Exp. 4, breaking the dormancy with ethylene chlorohydrin markedly increased the rooting at the cut surface induced by the subsequent treatment with neutralized indoleacetic acid.

#### EFFECT OF INDOLEACETIC ACID ON THE SPROUTING OF DORMANT OR FRESHLY-HARVESTED POTATO TUBERS

Although the experiments presented in the first part of this paper showed that indoleacetic acid retarded the sprouting of non-dormant pota-

to tubers, the possibility remained that it might act differently with dormant potato tubers and perhaps show a dormancy-breaking action. It soon became evident that the dormancy of potato tubers could not be broken with indoleacetic acid, but because of the difficulty of establishing a negative result conclusively, the experiments were continued to include many lots of tubers and a wide range of concentrations and times of treatment. In all cases the treatments were with the basal soak method at 10° C. which has been previously described. The pieces weighed about 20 g. and had one eye. For comparison and in order to show that the dormancy of each lot of tubers could be broken, the pieces were also treated with the dormancy-breaking chemical, ethylene chlorohydrin, 30 cc. per liter for 24 hours at 27° C. by the dip method of Denny (4). Following the treatments the pieces were planted 12 to a flat, usually in duplicate. A record of the number of pieces showing a sprout above ground was taken at intervals of three to seven days until all the pieces had either sprouted or rotted. From these data the average time to come above ground was found. Those cases in which more than two pieces out of 12 rotted are marked with an asterisk in Table XI.

Some of the details of these experiments are as follows:

Exps. 1 and 4. Irish Cobbler tubers from Hudson, New York.

Exp. 2. Bliss Triumph tubers from the Institute gardens.

Exp. 3. Irish Cobbler tubers from the Institute gardens.

Exp. 5. Small tubers of the Irish Cobbler variety from the Institute gardens.

Exp. 6. Irish Cobbler tubers from Rhinebeck, New York.

Exp. 7. Irish Cobbler tubers from Massachusetts.

Exp. 8. Russet Rural tubers from Pennsylvania.

Exp. 9. Irish Cobbler tubers from New Jersey.

Exp. 10. The same lot of tubers as in Exp. 9 was used two weeks later. The free acid was used instead of the potassium salt.

Exp. 11. An experiment similar to Exp. 9, but neutralized indolebutyric acid was used. The results were like those in Exp. 9 and are not given in the table.

The results given in Table XI showed that the only large effect of the treatments with the potassium salt of indoleacetic acid was to lengthen the time required to sprout when applied at the 200 and 800 mg. per liter concentration for 24 or 72 hours. This result showed definitely that the substance reached the buds and therefore also reached the buds in the lower concentrations, even though it had no marked effect on sprouting. The lack of marked stimulative action at the lower concentration, therefore, cannot be explained on the basis of lack of penetration. In a careful examination of the data for any slight stimulative action at the lower concentrations, the last column in Table XI was prepared by dividing the average sprouting



TABLE XI

EFFECT OF THE POTASSIUM SALT OF INDOLEACETIC ACID ON THE SPROUTING OF FRESHLY-HARVESTED POTATO TUBERS AS COMPARED WITH THE EFFECT OF ETHYLENE CHLOROHYDRIN

Treatment		Average time to come above ground in days							Ratio to control
Hrs.	K indoleace- tate mg., per liter	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 8	Exp. 6		
4	800.0	62	34, 31	83, 80	63, 56				1.15
	200.0	58	29, 26	75, 65	59, 51	49, 56	28, 23		1.01
	50.0	51	22, 23	90, 88	60, 60	54, 56	26, 34		1.04
	25.0	48					26, 25		0.82
	12.5	48	25, 28	95, 79	57, 68*	47, 45	30, 34		1.03
	3.13		26, 24	85, 90	58, 74	46, 55	33, 25		1.05
	0.78					56, 46			1.17
	0.20					44, 36			0.92
	0.05					46, 49			1.09
0.00 (H <sub>2</sub> O)	54	23, 21	72, 95	62, 55	42, 45	39, 29		1.00	
		Exp. 1	Exp. 5	Exp. 6	Exp. 7	Exp. 8	Exp. 9	Exp. 10†	
24	800.0	85	128, 100						1.48
	200.0	64	94, 94		40, 42	84, 88	71, 69	84, 79	1.31
	100.0	54			27, 31				0.96
	50.0	64	65, 52	31, 27	35, 30	76, 71	65, 60	47, 54	1.00
	25.0	58		39, 46	28, 29				1.03
	12.5	48	67, 66	34, 28	27, 23	58, 57	67, 64	52, 44	0.89
	3.13		90, 73	27, 31		56, 53	67, 65	50, 58	0.96
	0.78		75, 63	31, 29		52, 50	72, 66	51, 51	0.92
	0.20		74, 83	28, 39		42, 55			0.96
	0.05		67, 76			52, 61			1.03
	0.013		63, 77						0.88
0.00 (H <sub>2</sub> O)	55	69, 87	34, 41	21, 40	44, 47	78, 76	53, 53	1.00	
0.00 (H <sub>2</sub> O)		89, 76		31	53				
		Exp. 1	Exp. 2	Exp. 3	Exp. 4		Exp. 9	Exp. 10†	
72	800.0	115	114,* 172*	127, 120	139,* 127*				2.99
	200.0	84	56, 51	108, 97	75, 67		83, 78	99, 83	1.56
	50.0	63	27, 29	80, 86	61, 54		57, 71	50, 55	1.07
	12.5	54	26, 22	79, 76	55, 62		65, 71	43, 53	0.99
	3.13		25, 24	68, 85	56, 50		62, 60	56, 48	0.93
	0.78						62, 70	47, 47	0.94
	0.00 (H <sub>2</sub> O)	45	25, 24	83, 81	70, 61		69, 72	46, 54	1.00
		Exp. 1 and 4	Exp. 2	Exp. 3	Exp. 5	Exp. 6	Exp. 8	Exp. 9	
Ethylene chlorohy- drin dip		28	25, 22	17, 23	16, 16	13, 13	26, 22	19, 20	0.41
Control (H <sub>2</sub> O)		69	27, 31	77, 74	66, 79	36, 26	53, 68	65, 55	1.00

\* More than two pieces rotted without sprouting.

† The free acid was used instead of the potassium salt.

time for each treatment by the average sprouting time for the corresponding control and averaging these ratios. The results indicate a very slight stimulative action at 25 mg. for 4 hours, 12.5 mg. for 24 hours, and 3.13

mg. for 72 hours. Making use of the duplicate values, the standard deviation of a single determination amounts to six days. Using this value the odds that there is a significant stimulation at the above low concentrations are 3:1, 9:1, and 5:1 respectively, allowing for the fact that a number of treatments are compared with one control and the best selected as suggested by Tippet (15, p. 53). Such a small stimulation at a low concentration would be in agreement with a hypothetical curve for the action of auxin on bud growth advanced by Thimann (13). However, even if a stimulation at low concentration is conceded its magnitude is small and amounts to six days at the most. It has been observed in other experiments that many substances when applied at just the right concentration will hasten very slightly the sprouting of dormant potato tubers. The dormancy-breaking chemical, ethylene chlorohydrin, however, produced a marked stimulation and reduced the sprouting time 36 days. These results show that if indoleacetic acid is assumed to be equivalent to natural auxin, an assumption frequently made, these experiments offer little support for the idea that an increase of auxin-like substances in the tissue is the explanation for the breaking of dormancy of potato tubers.

#### EFFECT OF BREAKING THE DORMANCY OF POTATO TUBERS ON THE AUXIN CONTENT OF THE TISSUE

An obvious line of approach to the question of whether ethylene chlorohydrin breaks the dormancy of potato tubers by changing the auxin content of the tissue is to determine the auxin content of the tubers treated with ethylene chlorohydrin. A very satisfactory method for determining the auxin content of tissue extracts has been developed by Went (18) making use of the oat coleoptile. However, present methods for extracting auxin quantitatively from tissues are not very reliable, since too much effort has been expended on improving the final step of measurement of auxin and too little on finding how to extract it quantitatively from the tissue. The two methods of extraction that come nearest to being satisfactory appear to be the method of du Buy (5), making use of solid carbon dioxide and that of van Overbeek (16) in which pieces of surviving tissue are extracted with peroxide-free ether.

The most serious difficulty with the method of du Buy proved to be the frequent appearance of traces of chlorine in the commercial "Dry Ice" available locally. This is not a fault of the method, but does make it hard to use. Tank carbon dioxide frequently contains oil which interferes with the results. For these reasons it was necessary to discard a number of experiments in which no auxin was obtained from either the treated or untreated tissue. It was also necessary to modify the du Buy technic in order to obtain active extracts from the potato tissue.

Pieces were cut from beneath 24 eyes so that the total weight was 40 g.

The blocks of tissue were approximately cubical and the skin was sliced off in a thin layer, including any small sprout that might be present. This tissue was cut into thin slices and each slice dropped at once into a mortar containing enough solid carbon dioxide to quickly freeze the tissue and leave some excess. The frozen tissue was ground fine and 100 cc. of peroxide-free ether were added. The tissue was ground with the ether until thawing started. Solid carbon dioxide was added until the tissue pulp froze. The ether was decanted through a filter paper in a Büchner funnel. The frozen tissue was added back to the mortar, ground again with 100 cc. of ether, and this process repeated once more, using 300 cc. of ether in all, some of which evaporated. Any water was frozen out of the combined filtrates by adding solid carbon dioxide and the ether decanted into a 250 cc. flask and made to volume. A 125 cc. aliquot was evaporated for the oat coleoptile test.

In the van Overbeek procedure the tissue was cut in the same way as above, but only six pieces weighing 10 g. were usually used. These were sliced and dropped into 250 cc. of ether that had been treated with ferrous sulphate and calcium oxide and distilled to remove peroxides. The van Overbeek procedure was then followed. Aliquots representing 2.5 g. of tissue were evaporated for the oat coleoptile test.

The ether extract from either method was evaporated slowly on the steam bath to small volume, transferred to a small vial containing a small stirring rod, the total weight of which was known, and evaporated to about 0.2 cc. About 0.3 cc. of 3 per cent agar was added and the vial heated to evaporate the ether. The agar was well mixed with the stirring rod and cooled. Water was added to 0.3 g. and the agar melted, mixed, and poured into a brass mold. The agar blocks were cut  $2.7 \times 2.7 \times 1.5$  mm. and applied to oat coleoptiles, following the Went technic. For comparison, agar blocks containing 0.025 mg. of indoleacetic acid per liter gave a curvature of six degrees. The ethylene chlorohydrin treatments were by either the vapor or dip method of Denny (4) for 24 hours and one-eye pieces were planted until required for analysis.

The results are shown in Table XII. Each value in Table XII represents the average of five or more coleoptiles. It will be noted that treatment with ethylene chlorohydrin resulted in a large increase in auxin extracted by the modified du Buy technic. However, it appears that this may be due to the formation of auxin by the sprouting process, since in those cases where an increase in auxin was noted, the first evidence of sprouting was noticeable at the eyes of the treated pieces. In the determination made after three days, however, there were no signs of sprouting and also no increase in auxin. Bennett and Skoog (2) have noted an increase in auxin associated with the breaking of dormancy of the buds of fruit trees by low temperature treatment.

With the van Overbeek procedure the results were entirely different, no increase in auxin was observed and the amount of auxin extracted was much greater, since 2.5 g. of tissue yielded about as much auxin as 20 g. by the du Buy procedure in the treated samples and much more in the control samples. However, the higher values obtained by the van Overbeek procedure do not necessarily show that it is more nearly correct than the modified du Buy procedure, since it is essentially an ether extraction of surviving tissue, inasmuch as the sliced tissue is dropped into ether and may continue to produce auxin for some time. There is some evidence in favor of this, since if a neutralized solution of potassium cyanide is added

TABLE XII

EFFECT OF TREATMENT OF DORMANT POTATO TUBERS WITH ETHYLENE CHLOROHYDRIN ON THE AUXIN EXTRACTED FROM THE TISSUE BY DIFFERENT PROCEDURES

Method of extraction	Days after treatment	Average degrees bending of oat coleoptiles	
		Treated	Control
du Buy, modified 20 g. tissue	5	15	2
	5	10	2
	5	9	4
	4	10	1
	3	3	3
	7	11	1
van Overbeek 2.5 g. tissue	7	10	14
	7	12	14
	4	6	7
	7	10	12
	4	12	12
	7	8	12

to the ether, much less auxin is extracted. Also, if the ether is cooled to  $-75^{\circ}$  C. in a solid carbon dioxide-acetone bath before dropping in the tissue, somewhat lower results are obtained. Low results were also obtained when 1 cc. of 1 N HCl was added to the flask containing the ether and tissue.

Regardless of what effect ethylene chlorohydrin has on the auxin content of the tissue, the above results indicate that the auxin content of potato tubers is low. This is also in agreement with the lack of cut surface rooting of potato tissue unless it is treated with a root-inducing substance. The low content of auxin is evidence that the dormancy of freshly-harvested potato tubers is not due to the inhibiting effect of auxin on bud growth. However, this does not exclude the possibility that dormancy



might be due to an inhibiting substance which has no action on oat coleoptiles. It is interesting to point out that naphthaleneacetic acid has a very feeble action on oat coleoptiles as shown by Avery, Burkholder, and Creighton (1) so that the existence of substances inhibiting bud growth in low concentrations, but which would be missed by the oat coleoptile technic, is a possibility. Miller (11) has noted that treatment with solutions of thioacetamide inhibits the sprouting of potato tubers.

#### SUMMARY

1. The sprouting of the buds of non-dormant potato tubers was inhibited by treatment of cut pieces with solutions of 250 mg. or more of the potassium salt of 3-indoleacetic acid for two or more days at 10° C.

2.  $\alpha$ -Naphthaleneacetic acid inhibited the sprouting of cut pieces of potato tubers when applied in concentrations of 20 mg. or more of the potassium salt for one or more days. It was, therefore, more than ten times as effective as indoleacetic acid in inhibiting the growth of the buds of potato tubers.

3. Pieces of potato tubers, the sprouting of which had been inhibited by treatment with the potassium salt of naphthaleneacetic acid, could be made to sprout promptly by treatment with the dormancy-breaking chemical, ethylene chlorohydrin. However, the dormancy-breaking chemical, potassium thiocyanate, had little effect on the sprouting of such pieces.

4. The sprouting of the buds of whole potato tubers was inhibited by exposure to the vapor of the methyl or ethyl ester of naphthaleneacetic acid. This could be accomplished by storing the tubers in a container with filter papers impregnated with the ester. At temperatures of 22° and above, 2.5 mg. of the methyl ester of naphthaleneacetic acid per six tubers produced considerable inhibition of sprouting and 22 mg. or more produced marked inhibition. At 10° C. inhibition was evident with 22 mg. or more. The volatility of the methyl ester at 10° C. could be demonstrated by the production of epinasty of the leaves of tomato plants. The papers impregnated with the methyl ester could be used to inhibit the sprouting of a second lot of tubers. An exposure of 30 tubers for one day at 24° C. to 400 mg. of the methyl ester produced considerable inhibition of sprouting and exposure of seven days produced marked inhibition.

5. The buds of whole tubers, the sprouting of which had been inhibited with the vapor of the methyl ester of naphthaleneacetic acid, could be induced to sprout promptly by treatment with the vapor of ethylene chlorohydrin.

6. Exposure to the vapor of the methyl ester of naphthaleneacetic acid also retarded the withering of whole tubers.

7. Treatment with the vapor of acetonitrile produced considerable inhibition of the sprouting of whole tubers.

8. Treatment of cut pieces of potato tubers with the potassium salt of indoleacetic acid induced roots to grow at the cut surface of the tubers in from 11 to 24 days after treatment with 250 to 1000 mg. of the potassium salt per liter for periods of two hours to three days at  $10^{\circ}$  C. Roots could be induced to grow on pieces cut from the center of the tubers without skin or eyes.

9. Naphthaleneacetic acid induced roots to grow at the cut surface of potato tubers but less promptly than indoleacetic acid. However, it was active in much lower concentrations, producing rooting in about 40 days when applied in concentrations of 10 to 40 mg. of the potassium salt per liter for one to three days at  $10^{\circ}$  C.

10. Indoleacetic acid induced rooting sooner and more completely in old or non-dormant potato tubers than in freshly-harvested or dormant tubers.

11. Breaking the dormancy of freshly-harvested potato tubers with ethylene chlorohydrin caused them to root more readily when treated with indoleacetic acid.

12. Treatment of freshly-harvested potato tubers with ethylene chlorohydrin increased the amount of auxin extracted from the tissue by a modified du Buy procedure, but had no effect on the amount extracted by the van Overbeek method.

13. Indoleacetic acid did not break the dormancy of freshly-harvested potato tubers but inhibited sprouting when used in high concentrations. However, there was indication of a very slight stimulative action in the low concentrations. This was not more than six days while ethylene chlorohydrin reduced the average sprouting time 36 days.

14. Considered as a whole, the results of these experiments offer little support for the idea that the dormancy of potato tubers is regulated by increase or decrease of the amount of auxin-like substances in the tissue.

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# METHYLATION OF COTTON FIBER WITH ETHEREAL DIAZOMETHANE<sup>1</sup>

RICHARD E. REEVES AND H. JEANNE THOMPSON

As a part of studies on the structure of cellulosic fibers we are investigating the reasons why only a small proportion of the hydroxyl groups of cotton fibers is available for methylation with ethereal diazomethane. In the present report are described the preparation and properties of certain partly methylated fibers and also one of the factors which limit the reaction. Although several investigators have previously reported on the action of diazomethane on cotton fibers there appears to be no reference to the treatment of mercerized fibers with this reagent. The findings of previous workers are summarized in Table I.

TABLE I  
LITERATURE REFERENCES TO METHYLATION OF COTTON FIBER WITH DIAZOMETHANE

Material used	Solvent for diazomethane	% methoxyl	Reference
Cotton fiber	Ether	1.5-4.2	Geake & Nierenstein (7, footnote 3), Nierenstein (10)
Absorbent cotton dried in vacuum	Anhydrous ether	No appreciable reaction	Schmid (11)
Defatted cotton fiber	Ether	4.26	Fuchs & Horn (5)
Cotton and regenerated cellulose	Methanol	0.7-0.8	Lieser (8)

In the present investigation native cotton treated with ethereal diazomethane has given products containing considerably more methoxyl than has been reported by earlier workers, while mercerized cotton has been found to react more rapidly and to a greater extent than native fibers. Starting with raw cotton repeated treatment with diazomethane has given a product containing 9.8 per cent methoxyl, while mercerized cotton has taken up as much as 17.7 per cent (Table II). Completely methylated cellulose contains 45.6 per cent methoxyl.

As noted by Schmid (11) for starch and Brauns and Brown (2) for wood pulp, the methylation is dependent upon the presence of moisture. Thoroughly dried fibers do not react to an appreciable extent, which probably accounts for Schmid's failure to observe the methylation. No large

<sup>1</sup> Cellulose Department, Chemical Foundation, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York.

differences were observed in the methylation of mature cellulosic fibers from four different strains of cotton, spruce wood pulp, or flax. In contrast with the findings of Nierenstein (10), methylation in the presence of finely divided copper did not improve the reaction.

TABLE II  
REPEATED METHYLATION OF AIR-DRIED COTTON FIBER  
(0.5-0.9 M ETHEREAL DIAZOMETHANE)

Material used	No. of treatments								
	1	2	3	4	5	6	7	8	9
Raw cotton, % $\text{OCH}_3$	5.5	8.4	9.1	8.9	9.5	—	9.8	—	—
Merc. cotton, % $\text{OCH}_3$	11.7	13.6	14.5	15.1	15.8	17.3	17.8	—	17.7

All of the diazomethane methylation products retain their original fiber form. They do not disperse to an appreciable extent in sodium hydroxide solution, but disperse readily in cuprammonium hydroxide solution, showing viscosities approaching that of the unmethylated fibers from which they are derived. The optical rotations of the dispersions decrease with increasing methoxyl content. The partially methylated fibers show great resistance toward the action of cellulose decomposing bacteria. They give an X-ray diffraction pattern which differs from the diffraction pattern of a preparation containing approximately the same amount of methoxyl introduced by another method (Fig. 1).

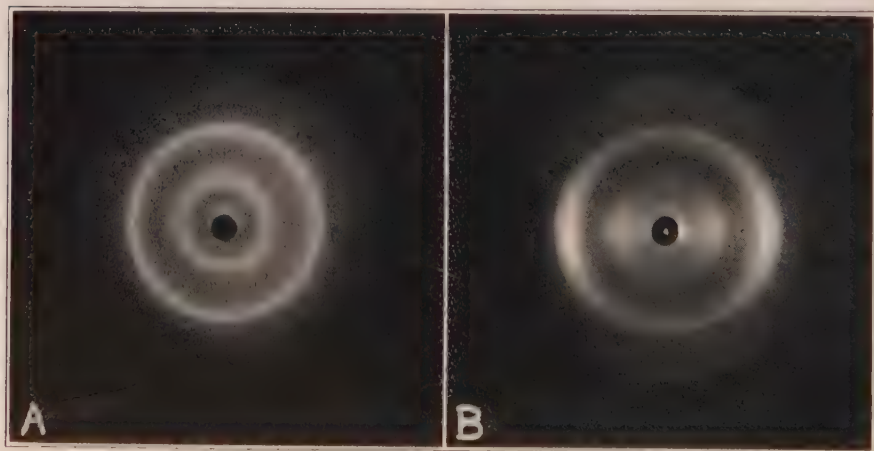


FIGURE 1. A. X-ray diffraction pattern of methyl cellulose containing 18.2 per cent methoxyl introduced by the action of dimethyl sulphate on cotton linters dispersed in dimethyldibenzylammonium hydroxide. B. X-ray diffraction pattern of mercerized cotton containing 17.7 per cent methoxyl introduced by means of ethereal diazomethane.

## EXPERIMENTAL PART

Unless otherwise noted, all experiments were made with mature fibers of *Gossypium hirsutum* L., Super Seven variety, Strain 4.

Diazomethane was prepared from nitrosomethylurea according to the directions in Organic Syntheses (4). The solutions were usually 0.5–0.9 molar with respect to diazomethane, which was determined by its reaction with benzoic acid (6, p. 162).

The methylations were carried out by allowing the cellulosic material to stand in the refrigerator at 0° to 5° C. for one week with a large excess of ethereal diazomethane in a flask bearing a capillary outlet tube for escape of gases. The concentration of diazomethane fell gradually to about 0.2–0.4 M during the course of a week. For the most highly methylated samples, several treatments were necessary. At the end of the final treatment, the fiber was washed with ether containing acetic acid, then with alcohol and water. All analytical values are calculated on the basis of anhydrous fiber. Micro methoxyl analyses were done according to the method of Niederl and Niederl (9, p. 191–192).

*Influence of moisture content of the fiber on the methylation.* When cotton which had been thoroughly dried was treated with diazomethane, it was found that only a very slight reaction took place with the anhydrous fiber, either native or mercerized. The results of experiments on fiber containing different amounts of moisture, as shown in Table III, indicate that the moisture content has a marked influence upon the course of the methylation.

TABLE III

INFLUENCE OF FIBER MOISTURE CONTENT ON THE METHYLATION (0.2 G. FIBER IN 20 CC. OF 0.5 M ETHERAL DIAZOMETHANE)

Pretreatment of fiber	Raw native fiber		Mercerized fiber	
	% moisture before methylation	% methoxyl after one treatment	% moisture before methylation	% methoxyl after one treatment
Dried in vac. at 100° C. over P <sub>2</sub> O <sub>5</sub>	0.00	0.25	0.00	0.48
Dried at room temperature over CaCl <sub>2</sub>	2.0	4.0	5.7	9.0
Air-dried	4.18	4.4	7.0	11.5
5 days in moist chamber	6.1	5.3	9.1	12.6

*Reaction with cellulosic fibers from different sources.* In order to investigate possible differences which might be exhibited by different strains of cotton, the following varieties in addition to Super Seven have been examined: *Gossypium barbadense* L., variety Pima, a long fiber, sea island cotton; *Gossypium hirsutum* L., variety Acala, a short fiber, upland variety; and *Gossypium hirsutum* L., variety Cleveland. All of these varieties are

grown commercially in the United States. In addition, tests were made on linen thread, spruce wood pulp,<sup>2</sup> and on immature fibers of Super Seven cotton from 25-day-old bolls. The results given in Table IV show remark-

TABLE IV  
METHYLATION OF VARIOUS CELLULOSIC FIBERS  
(0.2 G. AIR-DRIED FIBER IN 15 CC. OF 0.534 M ETHEREAL DIAZOMETHANE)

Fiber used	Native fiber, % methoxyl	Mercerized fiber, % methoxyl
Super Seven (mature)	5.5	12.2
Super Seven (from 25-day-old bolls)	7.5	12.1
Cleveland	5.4	12.2
Pima	5.5	11.4
Acala	5.7	12.0
Linen thread	4.6	11.9
Spruce wood pulp	7.0	12.8

ably little variation in methoxyl content among the mercerized fibers, while of the native fibers the immature Super Seven seems to have reacted to a greater extent than any other native fiber yet examined. However, the higher moisture content in the immature fiber (6.0 per cent compared with 5.0–5.3 per cent for the mature fibers) may account for this difference. Viscose rayon reacts to about the same extent as mercerized fiber.

*The viscosity and optical rotation of partially methylated mercerized cotton fiber dispersed in cuprammonium hydroxide.* Viscosities were determined at 25° C. in viscometers of the type recommended by Clibbens and Geake (3), slightly modified by the addition of ground glass stoppers in place of the rubber stoppers previously specified. The cuprammonium solution contained 15 g. of copper, 240 g. of ammonia, and 1 g. of sucrose per liter. Optical rotations were observed at 30° C. with the 0.4358  $\mu$  mercury blue line using an 0.5 dm. tube. The fiber concentration was 0.5 per cent in all cases. The observations are given in Table V.

TABLE V  
VISCOSITY AND OPTICAL ROTATION OF PARTIALLY METHYLATED MERCERIZED COTTON FIBER DISPERSED IN CUPRAMMONIUM HYDROXIDE

% methoxyl content of mercerized fiber	$\eta_{c.p.}$	$\alpha$
0	47.4	-3.00°
2.3	30.4	-2.65
6.5	29.8	-2.11
13.6	28.6	-1.79

<sup>2</sup> Methylation of wood or cellulosic material derived from wood with ethereal diazomethane has been reported by Fuchs and Horn (5), Brauns (1), and Brauns and Brown (2). Fuchs and Horn also refer to such work in the dissertation of Ungar (12).



## SUMMARY

Repeated methylation of mercerized cotton fibers with ethereal diazomethane has been found to yield products containing as much as 17.7 per cent methoxyl. Native cotton fibers were found to take up 9.8 per cent methoxyl, considerably more than has been introduced previously by this method.

Some of the properties of the methylated fiber have been described.

The moisture content of cotton fibers has a direct influence upon their reaction with diazomethane.

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# CELLULOSE MEMBRANES FROM VARIOUS PARTS OF THE PLANT KINGDOM<sup>1</sup>

FLORENCE L. BARROWS

The presence of cellulose in plants has been recognized for many years. Farr (10) has called attention to microchemical tests made in 1842 by Payen (28), who established the presence of cellulose in cell membranes; and to Valentin's (39) observations on the formation of cell walls from small particles.

The cell membrane in young living fibers of cotton, *Gossypium hirsutum* L., is built up of "visible anisotropic crystalline particles of uniform size in linear arrangement" (12, p. 200). These cellulose particles are ellipsoids about  $1.5\mu$  long and have refractive indices of 1.565 lengthwise and 1.530 crosswise. They are held together by a colloidal cementing material which contains some pectic acid (22). Mature cell membranes of cotton fibers are broken down by treatment with hydrochloric acid yielding separated cellulose particles (13). These particles give a typical X-ray pattern for cellulose (11).

More recent contributions (15) in this field have confirmed and extended the conception<sup>2</sup> that the cellulose particles form a crystalline discontinuous phase and the amorphous cementing material the continuous phase of the cellulose plant membrane. The cellulose particles are doubly refractive in polarized light. When treated with iodine and sulphuric acid the particles swell and turn blue. This reaction is the microchemical test for cellulose. Unlike sulphuric acid, phosphoric acid does not destroy the colloidal cementing material, which remains as a gelatinous coating over the surface of the cellulose particles (8, 9, p. 995, Plate II, Fig. 4).

## METHODS

Microscopic identification of the cellulose particles was made by their size, shape, and behavior in the process of membrane formation as observed in ordinary light, as well as by their double refraction in polarized light. The lenses employed were 20 X and 25 X oculars and apochromatic objectives 4 mm., N.A. 0.95, 46 X and 8 mm., N.A. 0.65, 23 X. Magnifications of about 900 were used for most of the studies. In polarized light, a 45 X objective, N.A. 0.65, was used most frequently. Photographic records were made with a simple type of camera and some of these photographs were later enlarged two or two and a half times.

Thin clear glass slides and No. 0 cover glasses of the best quality are

<sup>1</sup> Cellulose Department, Chemical Foundation, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York.

<sup>2</sup> Manuscripts in preparation in the Cellulose Department.

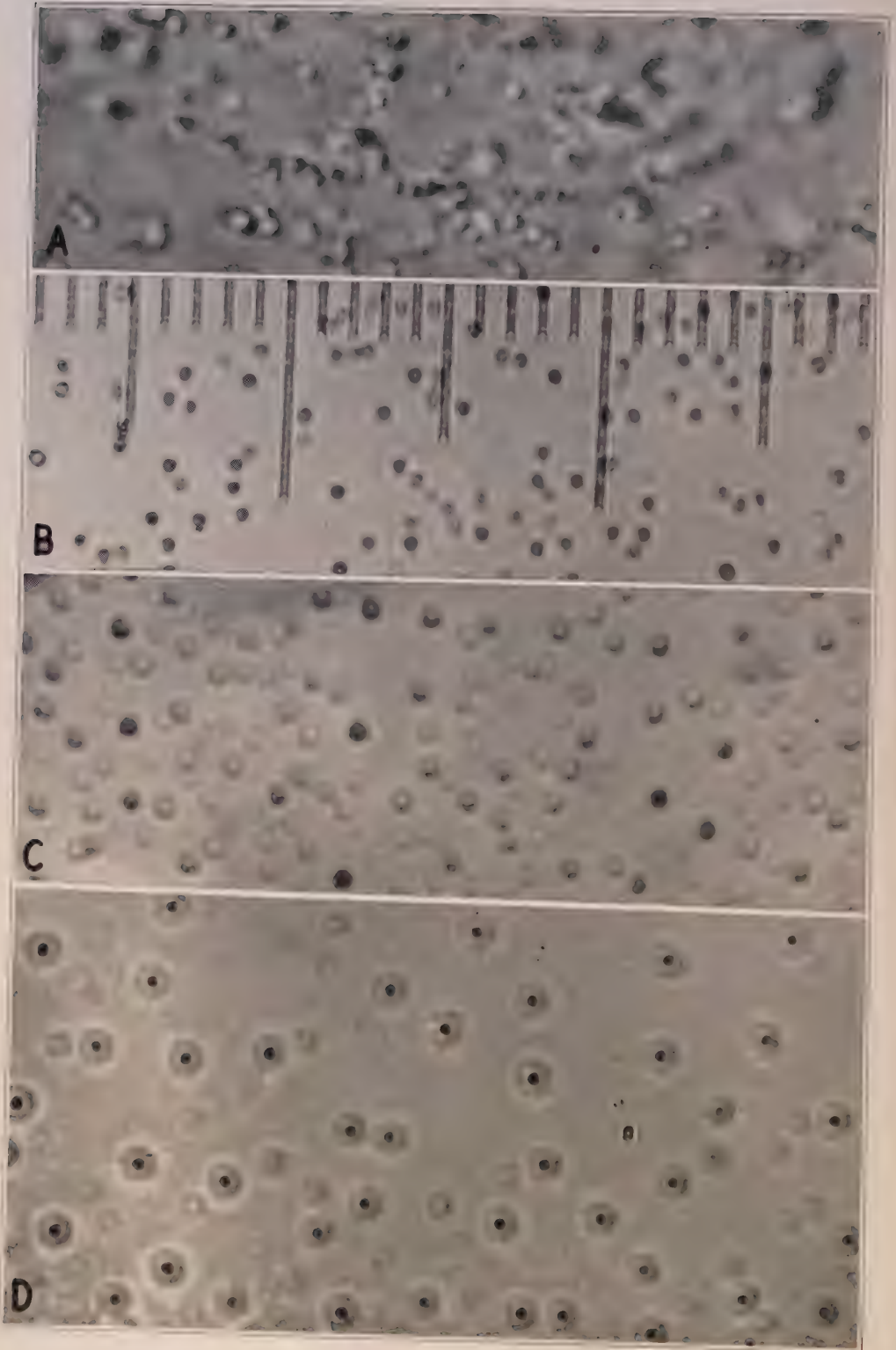


FIGURE 1. (For description see legend on opposite page.)



almost imperative for these observations. Square cover glasses are more convenient than circles for drawing reagents evenly through the mount with strips of filter paper.

Microchemical tests, as described by Farr and Eckerson (12), were used to identify the presence of cellulose in the particles. The iodine solution contained 1.5 grams of potassium iodide, and 0.3 grams of iodine to 100 cc. of distilled water. This solution was always added first, so that if starch grains were present, their position could be located. The excess solution of iodine in potassium iodide should be removed carefully by slowly drawing it through the slide with filter paper before adding the acid so as to prevent the formation of iodine crystals (1), which obscure the microchemical reactions.

*The cellulose reaction.* Sulphuric acid (75 per cent by volume) was added a drop at a time at one side of the cover glass. With a moist mount, or tissue mounted in water, the concentration is diluted below 75 per cent. Too high a concentration of acid may break down the cellulose structure so rapidly as to make it difficult or impossible to follow the swelling of individual particles. Under such drastic treatment, the membrane disintegrates into a cloudy blue mass. To prevent this rapid disintegration, the acid should be added slowly and drawn through the mount carefully with filter paper. Distilled water should be added to check the reaction when the desired stage has been reached (Fig. 1 B, C). Twenty-five per cent phosphoric acid may be used in place of sulphuric acid. For very delicate tissues the phosphoric acid is preferable as the reaction is slower and less violent and the cementing material is swollen but not destroyed (Fig. 1 D).

*Starch grains and cellulose particles.* Starch grains immediately turn blue in contact with iodine solution, while the cellulose particles are yellowish with their colloidal coating of cementing material and turn blue only after the addition of sulphuric or phosphoric acid. The cellulose particles are uniform ellipsoids and measure approximately 1.5 microns in length (12, p. 193), while starch grains are usually very much larger and show great variation in size and shape. In polarized light there is a marked difference between starch grains and cellulose particles. A dark cross can be seen on the starch grains, while the cellulose particles are bright. Reichert refers to the appearance of starch grains in polarized light as "an interference figure" or "cross" (32, p. 297). Other properties of starch

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FIGURE 1. Cellulose particles. A. *Cucurbita ficifolia* cortical cell of fruit. B. *Picea* bud +HCl to remove cementing material; +iodine and sulphuric acid; particles turning blue. One space on micrometer = 2.35  $\mu$ . C. *Vaccinium*, cranberry cortical cells of fruit +iodine and sulphuric acid; particles much swollen and turning blue. D. *Polytrichum* spores +iodine and phosphoric acid; slide dried four and one-half months. Each blue particle surrounded by swollen cementing material ( $\times 920$ , enlarged to 1840).

grains, such as the lower refraction, swelling in boiling water (32, p. 298) and in 10 per cent alkalis, serve to distinguish them from cellulose particles (11). After the iodine and sulphuric acid reaction, the size of the cellulose particles and the amount of swelling depend upon the length of treatment, the concentration of the acid, and the rate at which the reagents penetrate the cell membrane and the particles. (Compare the size of treated and untreated particles in Fig. 1 A and C.)

The number of cellulose particles in a given field depends largely upon the mechanical and chemical manipulations. The particles are so small that they may be lost or washed away by too rapid flow of reagents under the cover glass. They have a tendency to rise close to the under surface of the cover glass after treatment with sulphuric or phosphoric acid. Often a slide which has been allowed to stand a half hour, or even overnight, will contain many separated cellulose particles. By focusing just below the cover glass many free cellulose particles may usually be found.

It is better to use fresh living tissue, but cellulose particles can be identified in material fixed in formalin-alcohol either before or after imbedding in paraffin and sectioning. The fixing solution used was 960 cc. of 70 per cent alcohol plus 40 cc. of formalin. Farr has found 70 per cent alcohol with 1.4 per cent formalin best for cotton fibers and many other cells with which she has worked.

Plant cell membranes may be dissociated by appropriate chemical means so that the cellulose particles are set free from the cell wall. Dilute HCl (about 10 per cent) may be employed to remove the surface coating of cementing material if this is so dense as to obscure the microscopical and microchemical properties of the particles (13).

## CELLULOSE PARTICLES IN THE PLANT KINGDOM

### THALLOPHYTES

The plant kingdom offers a wide choice of material for the study of cellulose membranes. In the Thallophytes, both fungi and algae supply a great variety in membrane structure and composition.

*Fungi.* The presence of cellulose particles in the sporangiophores of *Aspergillus niger*, *A. ochraceous*, and *A. flavus* has already been reported by Farr and Eckerson (12, p. 194). In *Mucor* the cellulose particles are readily visible in young sporangiophores and sporangia (Fig. 2 A and C), and may be seen also in the living hyphae (Fig. 2 B). The spore wall of *Mucor* when mature contains one or more layers of cellulose particles. Spores of some species have a raised pattern, with cellulose particles in the membrane. As the spores germinate, the wall swells, increasing to several times the original size. The very tip of the germ tube as it emerges does not at first contain cellulose particles. As the young hypha develops cellu-

lose is soon deposited in the wall of the germ tube nearest to the spore coat, and cellulose particles also appear in the lumen. The walls of mature hyphae may have two or more layers of cellulose particles. At the bases of mature sporangiophores there may be several cellulose lamellae. In *Penicillium* sp. cellulose in the form of particles occurs in the mycelium as well as in the conidiophores and conidia. The common commercial mushroom (*Agaricus* Lin.) contains cellulose particles in more or less random arrangement in the hyphae found in the stipe, in the gill tissue, and in the basidiospores. A mycorrhizal fungus isolated from *Epigaea repens* L. contains cellulose particles in the membranes of the mycelium. The chlamydospores of this fungus have heavier walls than the spores of *Mucor*, and the cellulose particles contained in the lamellae can be clearly observed and identified.

The question of the presence of cellulose in fungi has received considerable discussion. Gilson (19, p. 420) thought all plant cell membranes, with the probable exception of fungi, contained cellulose. De Bary (4, p. 8) found cellulose in young cells of two species of *Mucor*, although he was unable to obtain the color reaction in mature stages. He called this substance "Pilzcellulose." Richter (33, p. 510) found de Bary's "Pilzcellulose" to be the same as common cellulose with an admixture of other materials. Using the iodine and sulphuric acid reaction, he identified cellulose in *Polyporus*, *Agaricus campestris*, and *Daedalea quercina*. The present observations confirm these earlier identifications of cellulose in fungi and show, in addition, that in the plants examined it is in the form of cellulose particles. Cellulose particles are not as numerous in these fungi as in many algae, but they are unquestionably present.

*Algae.* The presence of cellulose particles in *Spirogyra*, *Oedogonium*, and *Valonia* has already been reported by Farr and Eckerson (11, 12, p. 195-196). Farr demonstrated cellulose particles in *Chlamydomonas*, *Oedogonium* 9, p. 992-993, Plate I, Figs. 1, 2, and 3) and in *Valonia ventricosa* (9, p. 994-995, Plate II, Fig. 4). Photographs and X-ray diffraction patterns showed cellulose particles in *Valonia ventricosa* (11, p. 1130-1131, Plate III) and *Halicystis ovalis* (11, p. 1132-1135, Plates IV and V).

Cellulose particles can also be identified in the cytoplasm and in newly formed layers of the cell membranes of such algae as *Nitella*, *Cladophora*, *Vaucheria*, *Bulbochaete*, *Chlamydomonas*, *Oscillatoria*, *Chondrus crispus*, *Laminaria*, desmids, diatoms (Fig. 3), and many other forms. Some forms of *Bulbochaete* have setae which at their base are hollow cones narrowing to slender cylinders containing one layer of cellulose particles. These setae taper to a single row of cellulose particles surrounded by a coating of cementing material. The oogonia have heavy walls with several lamellae and the orientation of the cellulose particles is parallel to the surface of the protoplast. In some *Scenedesmus* colonies the projections have a similar



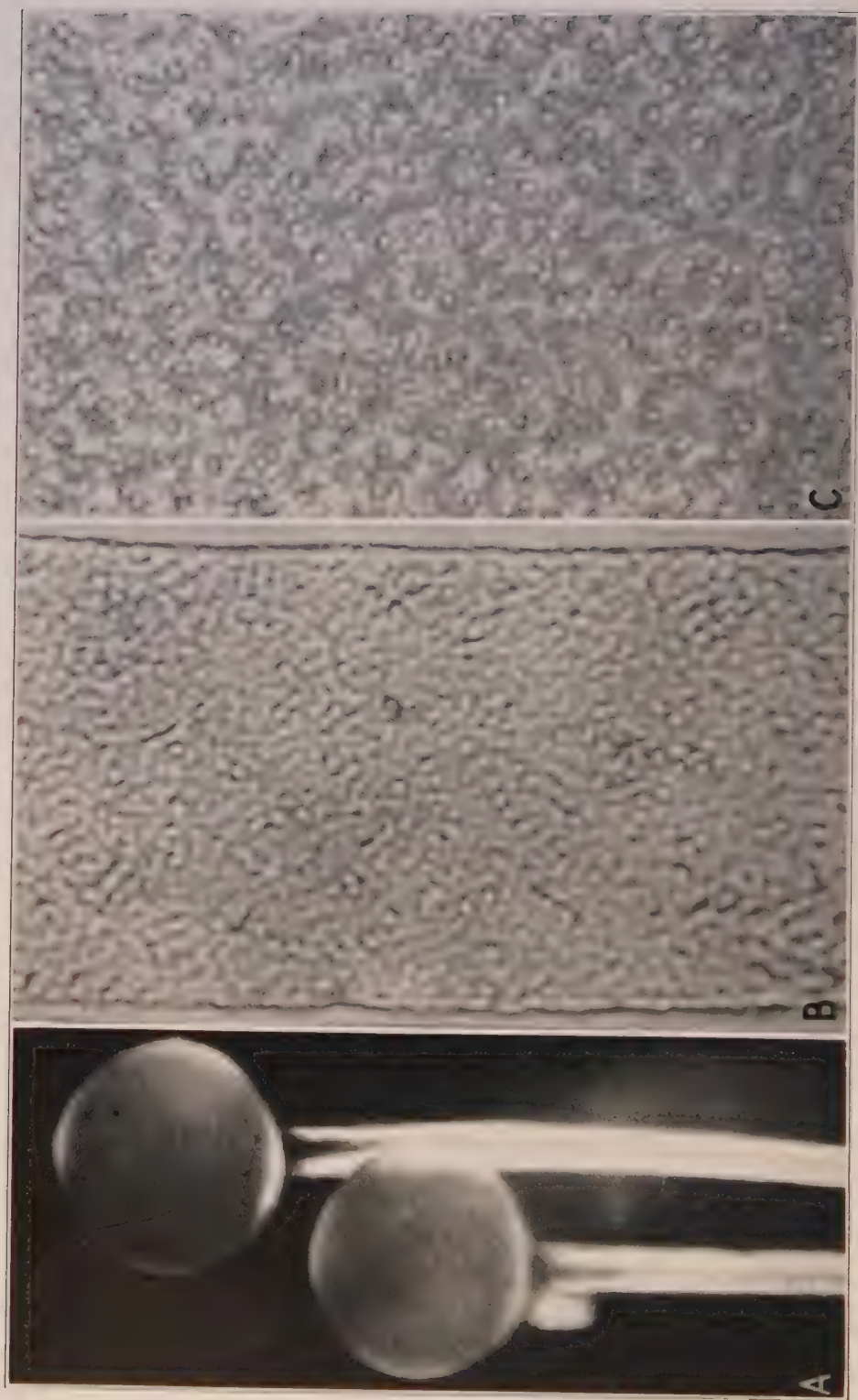


FIGURE 2. (For description see legend on opposite page.)



structure although they do not reach the extreme length of the setae found in *Bulbochaete*. In *Euglena* sp. the flagella contain a single row of cellulose particles in the basal end. These may extend one-half or two-thirds the length of the motile organ.

The opposite extreme in proportions of crystallites to cementing material is found in some of the diatoms, those minute one-celled plants the fossil skeletons of which have persisted from prehistoric geological periods. The living diatoms of today are often coated with a colloidal layer which stains with ruthenium red, but in dead or fossil cells much of this colloidal phase seems to have disappeared.

Diatoms have been reported to contain no cellulose in their cell walls (35, p. 129; 37, p. 195). Examination of several species of living diatoms shows the presence of cellulose particles not only in the cytoplasm but also regularly arranged in the cell wall (Fig. 3 A, D, E, F). Uniform-sized structures in mature diatoms have been frequently observed and illustrated (5, p. 67; 16, p. 34; 18, Plate 21; 21, Plates 3 to 9; 26, Plate 7, Fig. C and D; 31, Plate 9; 36, p. 137, Fig. 4; 38, Plate VI). Measurements made by Quekett (31, p. 508) and Fuge (17, p. 91) indicate that these "dots" or "puncta" are approximately a micron in size. The double refraction of these uniform-sized structures (Fig. 3 F) raises the question as to whether these "dots" or "puncta" may have resulted from the deposition of non-doubly refractive silicic material as a coating over cellulose particles during membrane formation.

Joan Kerr (24, p. 27), using "strong iodine" and 5 per cent sulphuric acid, has reported cellulose in the cell wall of *Pleurococcus vulgaris*. She has also found it in the cell wall of *Spirogyra* (25). Naylor and Russell-Wells (27, p. 640) have reported the presence of cellulose in the cell walls of four red algae and seven brown algae. Viel (40) has recently reported cellulose in two species of *Fucus* and two of *Laminaria*. Although Hassid (23, p. 462) has reported that *Iridaea laminarioides* (Rhodophyceae) "does not contain any cellulose," *Iridaea cordata*, a closely related species, when examined in our laboratory was found to contain cellulose particles in its walls.

#### BRYOPHYTES

*Liverworts.* The rhizoids of *Marchantia* contain large numbers of cellulose particles in the region of their actively growing tips. The lumen of the tip of the young rhizoid frequently contains dense masses of cellulose particles. The curious branches projecting into the cell lumen in certain rhizoids show the presence of crystalline material in polarized light. After

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FIGURE 2. Fungi. *Mucor*. A. Young sporangia, polarized light ( $\times 120$ , enlarged to 180). B. Hypha ( $\times 920$ , enlarged to 1840). C. Surface of sporangium ( $\times 920$ , enlarged to 1380).

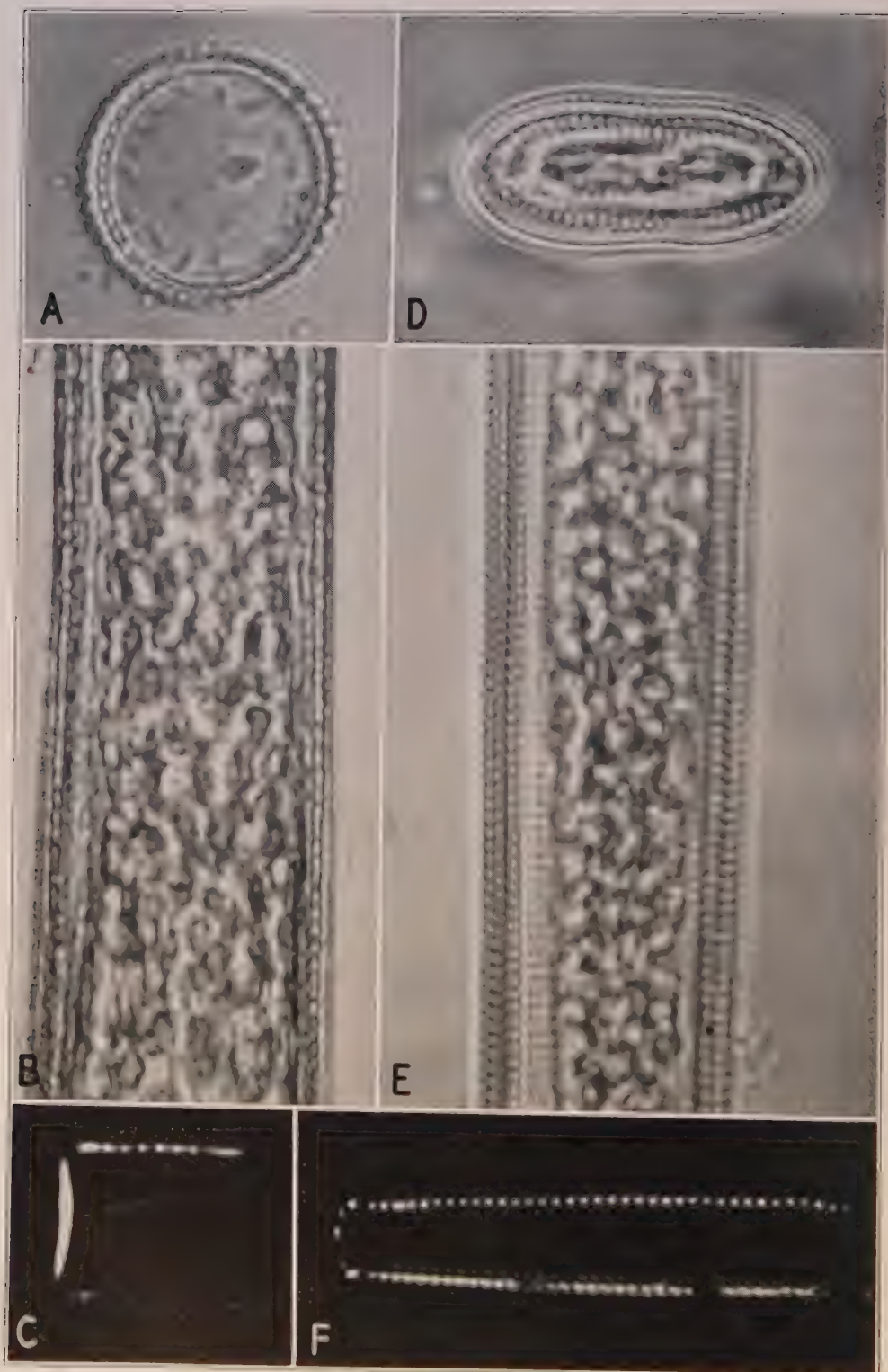


FIGURE 3. (For description see legend on opposite page.)

treatment with 10 per cent hydrochloric acid, the cellulose particles are clearly visible in these structures.

The sporophyte of *Anthoceros* contains cellulose particles in the spore mother cells and in the walls of the developing spore tetrads.

*Mosses.* Young leaves of *Mnium* (Fig. 4 A) show cellulose particles being laid down in the new walls of the mesophyll cells. At the left of the figure long rows of the particles may be seen lined up close to the wall. In these and other cells some short chains and irregular groups may be seen. The paraphyses of *Polytrichum*, the hair-cap moss, with their transparent walls are favorable material for observation (Fig. 4 B). The particles here have an orientation similar to that in the cells of the *Mnium* leaf. *Sphagnum* with its very peculiar leaf structure contains cellulose particles in both the thin- and thick-walled cells. *Sphagnum* leaves are equally interesting to study in polarized light, both with and without the selenite plate, due to the orientation of the cell walls at different angles.

Roberts and Haring (34) report cellulose in the spore walls of 20 species of mosses and have illustrated cellulose particles in the protonema of *Polytrichum commune* (34, Fig. 2). The legend of their Figure 2 calls attention to a line of cellulose particles in a cross wall, and also to the appearance of cellulose particles as observed while being laid down on the inner surface of the walls. This very thorough study of mosses employed both ordinary and polarized light observations with the microscope, as well as microchemical analyses for cellulose, pectic substances, lignin, cutin, and suberin (34, p. 102).

#### PTERIDOPHYTES

Among the fern allies, several species of *Lycopodium* have been found to have a resistant spore coat made up of three layers: (A) an outer waxy cuticle, (B) a middle silicious layer, and (C) an inner cellulose layer (3, p. 278-279).

Fern spores, which germinate much more readily than *Lycopodium*, have thinner and less resistant walls. The spores of *Onoclea sensibilis* L., the sensitive fern, contain cellulose particles in the enveloping membrane. Upon treatment with iodine and sulphuric acid, the thin outer wall is ruptured and portions slip off. The cellulose particles may be seen clearly in these outer layers. Further treatment with acid breaks down the membrane into short fibrils and chains and finally into separate cellulose particles. The growing tips of germinating fern spores, fern prothallia, tips of fern roots, root hairs (Fig. 5 A) and epidermal hairs of young fronds,

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FIGURE 3. Algae. A. Diatom ( $\times 900$ , enlarged to 1800). B. *Oedogonium* ( $\times 920$ , enlarged to 1840). C. *Oedogonium*, polarized light ( $\times 900$ , enlarged to 1800). D, E. Diatoms ( $\times 920$ , enlarged to 1840). F. Diatom, polarized light ( $\times 1125$ , enlarged to 2250).





FIGURE 4. (For description see legend on opposite page.)



*Dicksonia punctilobula* (Michx.) Gray, are favorable locations for studying the formation and structure of cellulose membranes.

#### SPERMATOPHYTES

*Gymnosperms.* The hard dense tissues of dry pine wood, *Pinus* sp.,<sup>3</sup> when treated with HCl gas and washed to neutrality, show fibrillar and particle structure on the broken surfaces and edges. When iodine and sulphuric acid are added, these fibrils break down to cellulose particles, separate and in chains. In *Picea* buds (Fig. 1 B) and in young living shoots of *Pinus nigra* Arnold and *Pinus resinosa* Ait., in the bud scales, young leaves and stem tissues, the particle structure of cellulose can be clearly seen.

*Angiosperms.* Among the angiosperms, epidermal hairs from various sources proved to be excellent material for observing the structure of the cellulose. Great variation was found in the thickness of the cell wall in trichomes of different ages from several genera. However, free cellulose particles are present in the lumen of most of them, especially in the younger cells. Typical examples are epidermal hairs from the young seed coat of cotton, *Gossypium hirsutum* L. var. Super Seven; hair from the flower stalk of common double red geranium, *Pelargonium* sp.; from the young stem of tomato, *Lycopersicon esculentum* Mill.; the down or fuzz from the young fruits of the peach, *Prunus persica* Sieb. & Zucc.; the epidermal hairs from young shoots of the stag-horn sumach, *Rhus typhina* L. (Fig. 5 B); the pappus of seeds of milkweed, *Asclepias*, or dandelion, *Taraxacum*; and in the multicellular hairs on young leaves of trailing arbutus, *Epigaea repens* L.

Stamen hairs and pollen of *Rhoeo discolor* Hance (Fig. 6 A) are especially favorable for observation where cellulose particles can be seen in the striae on the walls of young cells, as well as in the streaming cytoplasm. A recent photograph of *Rhoeo* pollen by Dermen (6, p. 216, Figs. J, M) shows clearly particles in the cell wall which are probably cellulose. The outer membrane of *Rhoeo* (Fig. 6 B), or *Zebrina pendula* Schnizl. filaments or anthers (Fig. 6 C) shows the particles in interesting patterns.

Streaming may be seen in the epidermal hairs from the corolla of *Cucurbita ficifolia* Bouché where the cellulose particles are carried about by the flowing cytoplasm. In order to secure photographs with a sharp focus, it is often necessary to add a little dilute iodine to check this motion.

<sup>3</sup> Sample supplied by the late Dr. Charles H. Herty from the Pulp and Paper Laboratory, Savannah, Georgia.

FIGURE 4. Bryophyta. A. *Mnium*. Edge of leaf. Cellulose particles in cell walls.  
B. *Polytrichum* paraphysis ( $\times 920$ , enlarged to 1840).

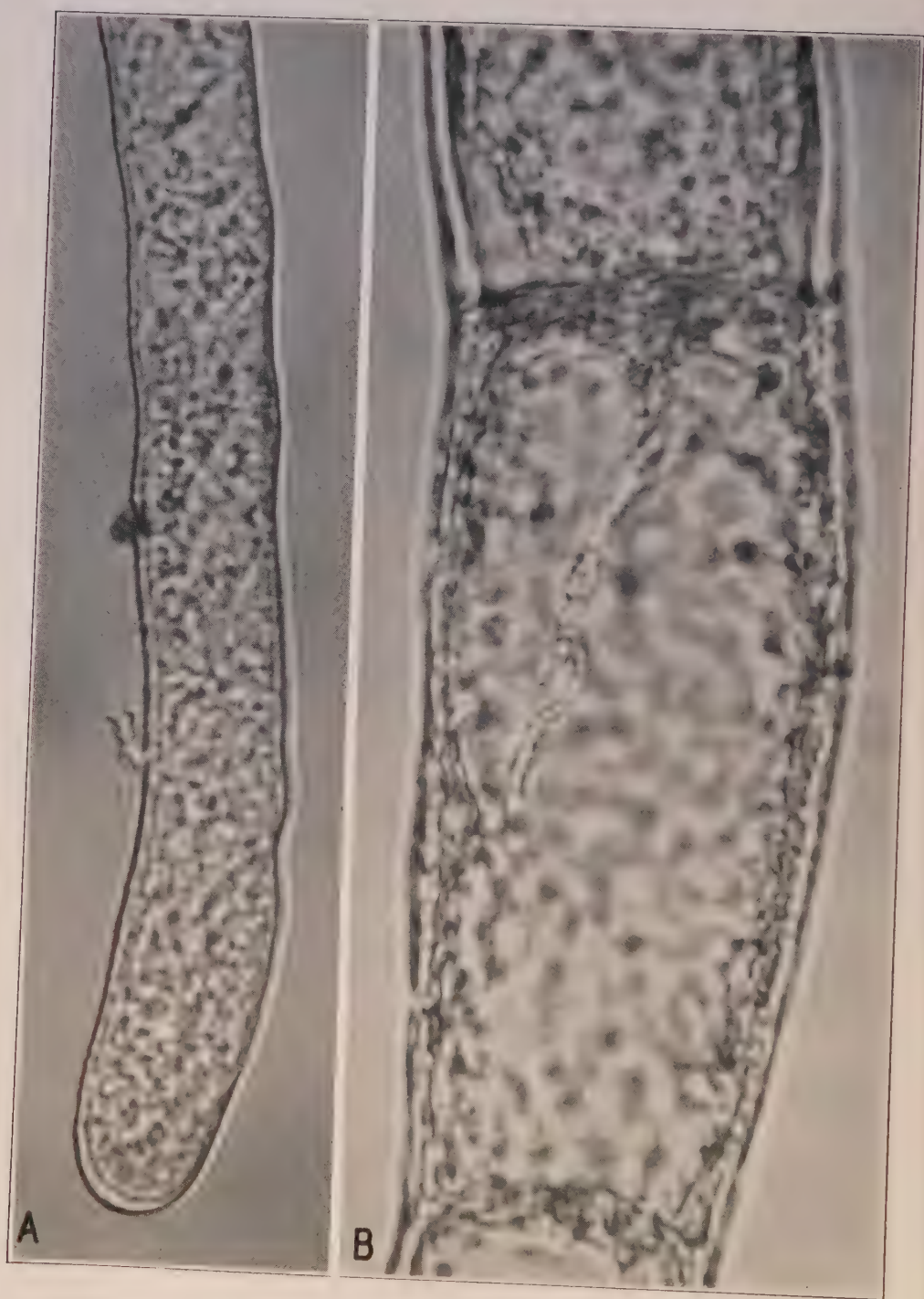


FIGURE 5. A. Pteridophyta. *Cyrtomium falcatum* Presl. Root hair on young sporeling ( $\times 900$ , enlarged to 1800). B. Spermatophyta. *Rhus typhina* L. Epidermal hair ( $\times 575$ , enlarged to 1437.5).

Roots are favorable regions for studying the presence and the properties of cellulose particles. The young roots of trailing arbutus, *Epigaea repens* L., are densely granular in the growing region where the cells are actively dividing and have not yet reached their full size. Magnifications around 900 show that this granular appearance is due to cellulose particles and their identity is confirmed by the iodine and sulphuric acid test. Plants which form root hairs, such as *Lilium regale* Wils., contain cellulose particles in the first bulge of the epidermis as root hair development is initiated. Root cap cells of *Epigaea repens* and *Cucurbita ficifolia* also contain cellulose particles. Pfeiffer has reported them in the roots of *Cissus sicyoides* L. var. *Jacquini* Planchon (29, p. 494). Priestley and Tupper-Carey (30, p. 219) claim that when the root meristem of *Vicia faba* L. is treated with iodine in potassium iodide and strong sulphuric acid "the walls of the meristem without exception show no trace of cellulose." Our observations do not confirm this. Their methods were rather drastic and may have removed the substance for which they were testing.

Fruit pulp of various kinds, such as the Malabar melon (*Cucurbita ficifolia*) shown in Figure 1 A, persimmon (*Diospyros* sp.), and cranberry (*Vaccinium macrocarpon* Ait.) shown in Figure 1 C, contains cellulose particles in cell walls and cytoplasm of the cortical region.

Cellulose particles may be observed in such leaves as the blanched heart of cabbage, *Brassica oleracea* L. var. *capitata* L., or in the cross sections of the midrib of celery leaves *Apium graveolens* L. var. *dulce* DC. They may also be found in the thin epidermis of the onion, *Allium cepa* L., the "silk" or style of sweet corn, *Zea mays* L. var. *rugosa* Bonaf., or in such hard tissue as the mature husk or pericarp of the shag-bark hickory nut, *Carya ovata* (Mill.) K. Koch.

In the light of these observations it would not be surprising that, if some of the plant materials which have been supposed to lack cellulose were observed more carefully microscopically by polarized light, and tested microchemically with iodine and sulphuric or phosphoric acid, at magnifications around 900, and with proper lighting, they might be found to contain cellulose particles.

#### ORIENTATION

In the 1934 paper of Farr and Eckerson (12), emphasis was placed on the end to end or linear arrangement of the cellulose particles in the formation of cell walls. Farr (11) has found that fibril formation in *Valonia* is quite similar to that in the cotton fiber. In the membranes of either of these plants the angle of orientation of the fibrils as measured by microscopic observations corresponds very closely to that found in the X-ray diffraction pattern. Farr and Sisson (14) have shown that the cellulose particles are arranged in transverse bands in the epidermal cells of *Avena*



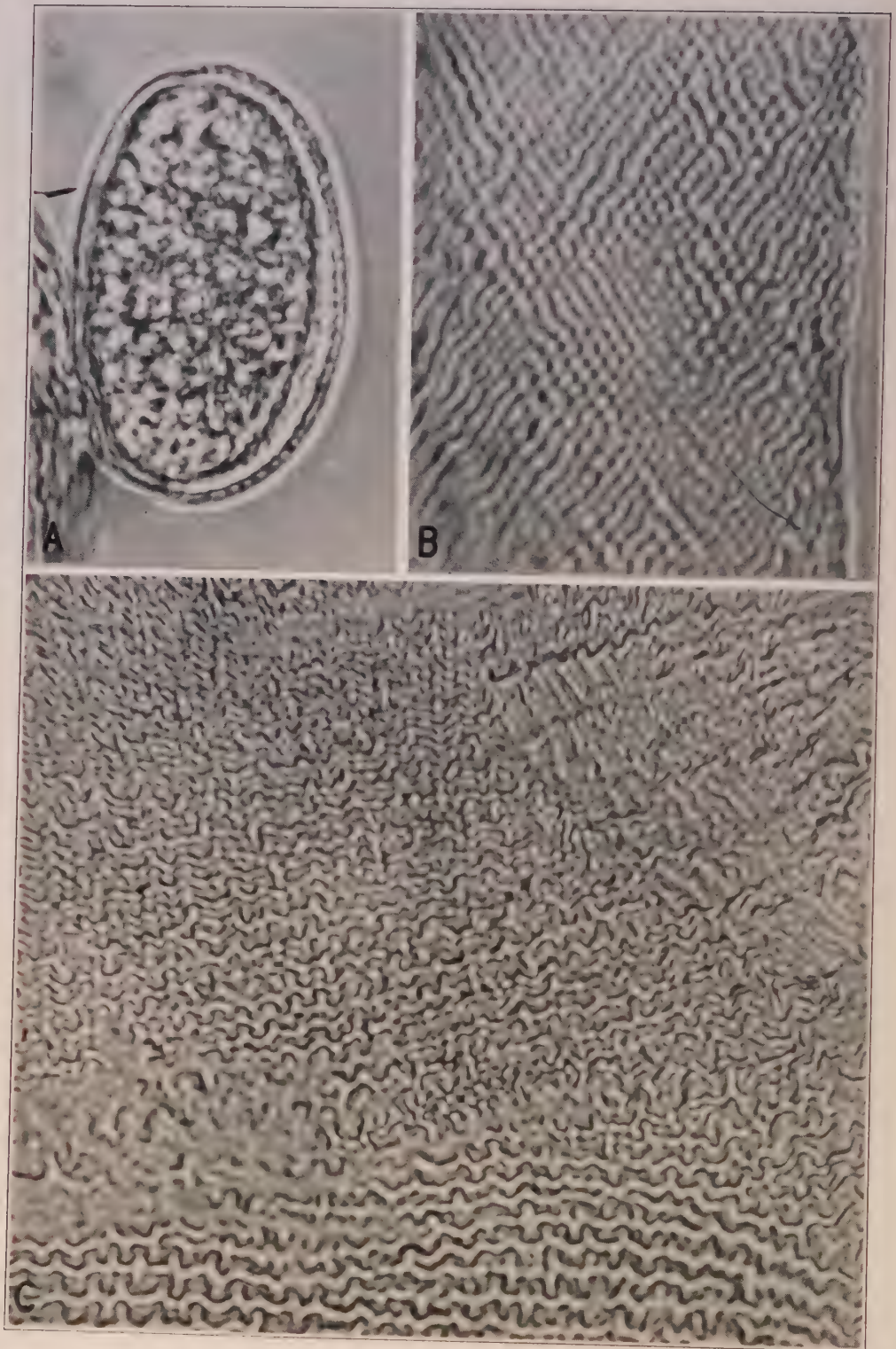


FIGURE 6. (For description see legend on opposite page.)



coleoptile. Here the long axis of the particles is oriented parallel to the long axis of the cell. The present study reveals that *Lilium* epidermis is very similar and is especially striking in polarized light. The long axis of the cellulose particle is usually parallel to the surface of the protoplast of the cell as Farr has already indicated (9, p. 988).

A single cell or a single plant with different tissues and organs may illustrate several types of orientation. In the present study it has been interesting to observe the orientation of the cellulose particles in many types of plant cells and tissues and to note the arrangement in cells of different geometric shapes. It is of great assistance to supplement ordinary microscopic observations with polarized light and X-ray diffraction studies.

Within the cytoplasm, the scattered cellulose particles in granular masses show little evidence of regular orientation. This is noticeable within the *Rhoeo* pollen grain (Fig. 6 A), or in the central cytoplasm of such other pollens as azalea (*Rhododendron*), arbutus, etc. The arrangement seems to be more or less characteristic of the spore walls of the common mushroom (*Agaricus*), and in the young sporangia walls of *Mucor* (Fig. 2 C). In the cytoplasm of cortical cells in fruits of such various plants as the Malabar melon (Fig. 1 A), persimmon, black alder, apple, etc., the cellulose particles show little orientation. Particles in the lumen of such cells as the root caps of cucurbits and arbutus, root hairs of ferns (Fig. 5 A), and the epidermal hairs of many plants show a similar arrangement. The densely granular cytoplasm of meristematic tissues and young growing regions of numerous plants show this lack of definite orientation.

While this type of arrangement of the cellulose particles is general within the cytoplasm, evidence of definite orientation may be observed in the outer regions close to the developing cell wall. Short chains of particles often occur adjacent to the plant membrane as observed by Farr in *Valonia* (11, p. 1131, Plate III, Fig. 1) and cotton (12, p. 202, Fig. 1 e). In the present survey 18 to 20 particles in one chain have been counted in epidermal hairs on a young peach fruit. Such chains in linear arrangement may also be found in young milkweed hairs. In many long plant cells the orientation of these chains of particles seems to be more or less parallel to the long axis of the cell as in moss leaves (Fig. 4 A) and moss paraphyses (Fig. 4 B). This parallel arrangement is particularly striking in *Oedogonium* (Fig. 3 B and C) and in some diatoms (Fig. 3 E and F). The particles in adjacent rows are frequently staggered, so that the widest part of a given particle fits into the space where adjoining particles are in contact at their rounded ends (Fig. 3 E). In other diatoms, the particles are arranged in

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FIGURE 6. Spermatophyta. Monocotyledons. A. *Rhoeo discolor* Hance. Pollen ( $\times 460$ , enlarged to 920). B. Epidermis of anther. C. *Zebrina pendula* Schnizl. Epidermis from anther ( $\times 920$ , enlarged to 1840).

horizontal as well as parallel rows. On cross walls or the ends of cylindrical cells (Fig. 5 B), the particles may have their long axes at right angles to the long axes of the cells as seen in the left end of the *Oedogonium* and the diatom in Figure 3 C and F. This still maintains the long axes of the particles parallel to the surface of the protoplast. Not all cross walls form a right angle with the long axis of the cell, so angles of various degrees may be observed. The cellulose particles in such walls naturally follow the angle of the wall in more or less linear arrangement.

In the cuticle and epidermis of *Zebrina*, a curious zigzag or herringbone pattern is formed (Fig. 6 C). In some of the diatoms with elaborate surface patterns on the valves, orientations at various angles and curves may be found.

In some of the highly differentiated types of cells such as the cotton fiber, the linear rows of cellulose particles become fibrils with extremely firm end to end attachment of the particles. These fibrils are arranged in a spiral around the long axis of the cell. Kapok fibers at the base show a spiral arrangement, but in the upper portion of the hair, orientation is much more frequently parallel than in cotton. In cylindrical hyphae of the mycelium of *Mucor*, orientation is nearly parallel to the long axis, while in more highly specialized structures like the mature sporangiophores of both *Mucor* and *Aspergillus* the arrangement is definitely spiral. Pine wood shows spiral orientation in the xylem cells. In bordered pits of conifers, the cellulose particles are arranged in concentric circles as is brought out clearly by rotating them in polarized light with a selenite plate.

In spherical cells of some of the unicellular algae, or cells with curved surfaces which approach the spherical, the cellulose particles follow the periphery of the cell. A spherical diatom in median focus (Fig. 3 A) illustrates this arrangement which resembles a string of beads lying in a circle. A median optical view of certain pollen grains, such as *Rhoeo discolor* (Fig. 6 A), shows a similar arrangement on curved surfaces. Adjacent rows of particles or concentric lamellae on such curved surfaces frequently show the same staggered arrangement as previously mentioned for parallel rows on cylindrical or rectangular faces of cellulose membranes.

#### PLANTS WHICH CONTAIN CELLULOSE PARTICLES

The following is a partial list of plants which have been examined and have been found to contain cellulose particles. Cellulose particles are not limited to the tissues listed below, but may occur in other portions of the plants as well. These are merely suggestive as regions in which the particles may be readily identified. The lower plants are listed according to Engler and Prantl (7). The higher plants are named according to the manuals of Gray (20) and L. H. Bailey (2).

## I. THALLOPHYTA

A. *Fungi*

Family	Genus	Tissue
Agaricaceae	<i>Agaricus</i> Lin.	Stipe, gills, basidiospores
Aspergillaceae	<i>Aspergillus</i> Micheli	Sporangiophores
	<i>Penicillium</i> Link	Mycelium, spores
Mucoraceae	<i>Mucor</i> (Micheli) Link	Mycelium, sporangio- phores, spores
Mucedinaceae (?)	Mycorrhizal fungus iso- lated from <i>Epigaea re- pens</i> L.	Mycelium, chlamydo- spores

B. *Algae*

Oscillatoriaceae	<i>Oscillatoria</i> Vaucher	Cell walls and cytoplasm
Euglenaceae	<i>Euglena</i> Ehrenb.	" " " "
Bacillariaceae	<i>Navicula</i> Bory and sev- eral other genera	" " " "
Desmidiaceae	<i>Closterium</i> Nitzsch.	" " " "
	<i>Cosmarium</i> (Corda) Lund	" " " "
Zygnemaceae	<i>Spirogyra</i> Link	" " " "
Volvocaceae	<i>Chlamydomonas</i> Ehrenb.	" " " "
	<i>Sphaerella</i> Sommerf.	" " " "
Pleurococcaceae	<i>Pleurococcus</i> Menegh.	" " " "
	<i>Scenedesmus</i> Meyen	" " " "
Hydrodictyceae	<i>Hydrodictyon reticulatum</i> (L.) Lagerh.	" " " "
Ulvaceae	<i>Ulva lactuca</i> Wulf	" " " "
Ulothricaceae	<i>Microspora</i> (Thur.) Lagerh.	" " " "
Oedogoniaceae	<i>Oedogonium</i> Link	" " " "
	<i>Bulbochaete</i> Ag.	" " " "
Coleochaetaceae	<i>Coleochaete</i> Breb.	" " " "
Cladophoraceae	<i>Cladophora</i> Kutz.	" " " "
Vaucheriaceae	<i>Vaucheria</i> DC.	" " " "
Valoniaceae	<i>Valonia ventricosa</i>	" " " "
	<i>Ihlicystis ovalis</i> (Lyngb.) Aresch.	" " " "
Characeae	<i>Nitella</i> Ag.	" " " "
Laminariaceae	<i>Laminaria</i> Lamx. 2 sp.	Stipe and thallus cell walls
Gigartinaceae	<i>Chondrus crispus</i> (L.) Stackh.	Cell walls
	<i>Iridaea cordata</i>	" "

<i>Ceramiales</i>	<i>Microcladia coulteri</i>	Cell walls
<i>Corallinales</i>	Unidentified red alga	" " and cytoplasm

## II. BRYOPHYTA

A. *Hepaticales*—Liverworts

<i>Marchantiaceae</i>	<i>Marchantia</i> (L.) Raddi	Rhizoids, thallus, gemmae
<i>Anthocerotaceae</i>	<i>Anthoceros</i> L.	Spore mother cell, tetrads

B. *Musci*—Mosses

<i>Sphagnaceae</i>	<i>Sphagnum</i> (Dill.) Ehrh.	Leaves, paraphyses, spores
<i>Mniaceae</i>	<i>Mnium</i> (Dill. ex p.) L. emend.	Leaves, stems
<i>Polytrichaceae</i>	<i>Polytrichum</i> Dill.	Leaves, stems, para- physes, spores

## III. PTERIDOPHYTA

<i>Polypodiaceae</i>	<i>Asplenium platyneuron</i> (L.) Oakes	Germinating spores, spo- rangium
	<i>Cyrtomium falcatum</i> Presl.	Prothallus, rhizoids, sporeling leaf and root
	<i>Dicksonia punctilobula</i> (Michx.) Gray	Epidermal hairs
	<i>Onoclea sensibilis</i> L.	Sporangia, spores, pro- thallus
	<i>Lycopodium annotinum</i> L.	Spore wall, stem tissue
<i>Lycopodiaceae</i>	<i>Lycopodium clavatum</i> L.	" " " "
	<i>Lycopodium complanatum</i> L. var. <i>flabelliforme</i> Fernald	" " " "
	<i>Lycopodium lucidulum</i> Michx.	" " " "
	<i>Lycopodium obscurum</i> L.	" " " "
<i>Selaginellaceae</i>	<i>Selaginella</i> sp.	Leaves, roots

## IV. SPERMATOPHYTA

A. *Gymnosperms*

<i>Pinaceae</i>	<i>Pinus nigra</i> Arnold	Young leaves, bud scales, stem
	<i>Pinus resinosa</i> Ait.	" " " "
	<i>Picea canadensis</i> (Mill.) BSP.	Bud, 1-year wood



B. *Angiosperms*1. *Monocotyledons*

<i>Gramineae</i>	<i>Zea mays</i> L. var. <i>rugosa</i> Bonaf.	Style or "silk"
	<i>Avena sativa</i> L.	Coleoptile, root hairs, leaves
<i>Commelinaceae</i>	<i>Commelina coelestis</i> Willd.	Epidermis, anthers, pollen, stem
	<i>Zebrina pendula</i> Schnizl.	Epidermis, stamen hairs, pollen
	<i>Tradescantia virginiana</i> L. var. Heavenly Blue	" " " "
<i>Liliaceae</i>	<i>Rhoeo discolor</i> Hance	" " " "
	<i>Lilium regale</i> Wils.	Roots
	<i>Lilium longiflorum</i> Thunb. var. <i>eximium</i> Nichols.	Leaf epidermis, peduncle, cell wall
	<i>Allium cepa</i> L.	Epidermis of bulb scales

2. *Dicotyledons*

<i>Juglandaceae</i>	<i>Carya ovata</i> (Mill.) K. Koch	Husk or pericarp
<i>Fagaceae</i>	<i>Castanea dentata</i> (Marsh.) Borkh.	Decaying wood
<i>Loranthaceae</i>	<i>Phoradendron flavescens</i> (Pursh) Nutt.	Fruit
<i>Caryophyllaceae</i>	<i>Lychnis coronaria</i> Desr.	Epidermal hairs, epidermis of peduncle, calyx, pollen
<i>Ranunculaceae</i>	<i>Paeonia suffruticosa</i> Andr.	Pollen, flower
<i>Cruciferae</i>	<i>Brassica oleracea</i> L. var. <i>capitata</i> L.	Leaves
	<i>Brassica alba</i> Ratenh.	Seed
<i>Crassulaceae</i>	<i>Sedum acre</i> L.	Leaves, epidermis
<i>Saxifragaceae</i>	<i>Philadelphus</i> sp.	Pollen
<i>Rosaceae</i>	<i>Prunus persica</i> Sieb. & Zucc.	Epidermal hairs of fruit
	<i>Pyrus malus</i> L.	Fruit pulp cortical cells
	<i>Crataegus</i> sp.	" " " "
<i>Leguminosae</i>	<i>Vicia faba</i> L.	Meristem of root and shoot in seed
<i>Tropaeolaceae</i>	<i>Tropaeolum majus</i> L.	Seed, cell walls of endosperm
<i>Linaceae</i>	<i>Linum usitatissimum</i> L.	Bast fibers of stem

<i>Geraniaceae</i>	<i>Pelargonium hortorum</i> Bailey	Epidermal hairs of young stem
<i>Euphorbiaceae</i>	<i>Ricinus communis</i> L.	Stem, bast fibers
<i>Anacardiaceae</i>	<i>Rhus typhina</i> L.	Epidermal hairs of young stem
<i>Aquifoliaceae</i>	<i>Ilex verticillata</i> Gray	Fruit pulp cortical cells
<i>Malvaceae</i>	<i>Gossypium hirsutum</i> L. var. Super Seven	Epidermal hairs of seed coat, stem tissue, petiole, pollen
	<i>Gossypium hirsutum</i> L. var. Acala	Stem, root, leaves, epidermal hairs
<i>Bombaceae</i>	<i>Ceiba pentandra</i> Gaertn.	Fibers from capsule
<i>Umbelliferae</i>	<i>Apium graveolens</i> L. var. <i>dulce</i> DC.	Petiole, leaves
<i>Ericaceae</i>	<i>Monotropa uniflora</i> L.	Scale leaves, pollen
	<i>Rhododendron calendulaceum</i> Torr.	Pollen, pistil
	<i>Epigaea repens</i> L.	Roots, seed coat, germinating seeds, epidermal hairs, leaves
	<i>Epigaea asiatica</i> L.	Roots
	<i>Vaccinium macrocarpon</i> Ait.	Fruit
<i>Ebenaceae</i>	<i>Diospyros</i> sp.	Fruit pulp
<i>Asclepidaceae</i>	<i>Asclepias tuberosa</i> L.	Pappus of seeds
	<i>Asclepias syriaca</i> L.	" " "
<i>Convolvulaceae</i>	<i>Cuscuta</i> sp. on <i>Hedera helix</i> L.	Stem, cortex, epidermis
<i>Solanaceae</i>	<i>Lycopersicon esculentum</i> Mill.	Epidermal hairs
<i>Scrophulariaceae</i>	<i>Verbascum blattaria</i> L.	Epidermal and glandular hairs
<i>Cucurbitaceae</i>	<i>Cucurbita pepo</i> L. var. <i>condensa</i> Bailey	Pollen, peduncles, petioles, hairs
	<i>Cucurbita ficifolia</i> Bouché	Fruit pulp, cortex, corolla, pollen, epidermal hairs, veins
<i>Compositae</i>	<i>Taraxacum officinale</i> Weber	Pappus of seeds
	<i>Lactuca sativa</i> L.	Leaves, seeds

From this list it is clear that cellulose, in the form of uniform-sized, ellipsoid, crystalline particles, is widely distributed throughout the plant

kingdom. The extension of the list awaits time for further observations and analyses.

#### SUMMARY

Cellulose, in the form of ellipsoidal crystalline particles about a micron in size, has been identified in the cytoplasm and cell membranes of a number of plants belonging to the Thallophytes, Bryophytes, Pteridophytes, and Spermatophytes. They are especially numerous in young growing regions, where they are found in the cytoplasm as well as in various stages of orientation in outer regions of the protoplast. Mature membranes in which these individual structural units are no longer visible break down upon treatment with hydrochloric acid (sp. gr. 1.19, room temperature) to reveal their component cellulose particles. The non-crystalline cementing material constitutes the continuous phase of the membranes, and the cellulose particles the discontinuous phase in all of the cellulose membranes examined.

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## TESTS ON CERTAIN ORGANIC COMPOUNDS FOR CONTROL OF ADULT JAPANESE BEETLE<sup>1,2</sup>

ALBERT HARTZELL AND FRANK WILCOXON

During the summer of 1938, an opportunity presented itself to conduct some preliminary tests on a number of combinations of organic compounds (1) as possible contact insecticides for the control of the Japanese beetle (*Popillia japonica* Newm.). Certain phases of the work were repeated during the summer of 1939. The experimental procedure consisted in spraying infested rose bushes in the field with a given spray solution, and immediately removing the sprayed beetles to field cages. Counts of living and dead adults were made of the caged beetles at intervals of 24, 48, and 72 hours. The results appear in Table I. An adequate amount of fresh rose blooms was supplied daily to caged beetles in order to remove the factor of starvation. As soon as a spray solution was made up, it was applied by means of a hand sprayer. Sprays were applied at intervals during the period of July 19 to September 2, 1938, and in August, 1939. Duplicate sprayings were made on the same day.

In order to prepare an effective pyrethrum spray it is necessary to have both a suitable solvent and a suitable wetting agent. Preliminary tests had shown that a solvent consisting of an aqueous solution of  $\beta$ ,  $\beta'$ -dichloroethyl ether with a sulphated alcohol (4) as a wetting agent (Tergitol 7 penetrant) were not very effective (58 per cent kill in 48 hours) in the control of adult Japanese beetles. Mixtures of solvents such as mesityl oxide, methyl butyl ketone, and  $\beta$ ,  $\beta'$ -dichloroethyl ether with a wetting agent (Tergitol 7 penetrant) were also unsatisfactory as well as a solution of ethylene glycol with Tergitol 7 penetrant (Table I). Various combinations of pyrethrum extract with Tergitol 7 penetrant and with other compounds (methyl isobutyl ketone and acetone) were tested. It was found that Tergitol 7 penetrant could act both as a solvent for pyrethrum resins and as a wetting agent, and that excellent results (100 per cent kill) could be obtained with a spray consisting of Tergitol 7 penetrant (0.5 per cent), and pyrethrum resins (0.1 per cent resins, total pyrethrins 0.02 per cent) with no other organic compound (Table I). It is necessary that the spray be thoroughly agitated during mixing and application. The Japanese beetle adults that were used in the above test were collected from unsprayed roses in the field. The possibility that the adults had fed previously

<sup>1</sup> Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 206.

<sup>2</sup> The materials for this investigation were furnished by the Carbide and Carbon Chemicals Corporation, New York, N. Y.

on sprayed foliage could not be eliminated. It was decided to compare these results with those obtained from beetles reared under conditions where the possibility of contact with insecticides would be avoided. Accordingly an area of untreated lawn heavily infested with grubs was caged in the spring of 1939 before the grubs had migrated to the surface. Adult beetles collected from this cage were used in tests made during the summer of 1939. The kills averaged 85 per cent, which was somewhat lower than the results obtained the previous year from beetles collected in the field, but may still be regarded as satisfactory for an insect as resistant as the adult Japanese beetle.

TABLE I  
TOXICITY TO ADULT JAPANESE BEETLE OF ORGANIC CONTACT SPRAYS

% conc. of spray mixture	Replicate counts	
	No. beetles	% dead in 72 hrs.
0.1% pyrethrum resin* + 1.0% acetone + 0.5% Tergitol 7 penetrant	73	93.2
	70	94.1
0.5% Tergitol 7 penetrant	115	64.3
	124	58.1
1.0% acetone + 0.1% pyrethrum resin*	76	1.0
	65	7.0
0.5% Tergitol 7 penetrant + 0.1% pyrethrum resin*	101	100.0
	113	100.0
0.1% pyrethrum resin* + 0.1% methyl isobutyl ketone + 0.5% Tergitol 7 penetrant	124	90.3
	111	99.1
Control—natural mortality	100	5.8
	74	5.4

\* 20 per cent total pyrethrins.

#### TOLERANCE TO PLANTS

The following 26 species of plants were tested: *Aesculus hippocastanum* L. (horse-chestnut), *Althea rosea* Cav. (hollyhock), *Antirrhinum majus* L. (snapdragon), *Betula pendula* Roth var. *Tortuosa* (white birch), *Corylus* sp., *Dahlia* sp., *Gladiolus* sp. vars. Purple Glory and Senorita, *Hedera helix* L. (English ivy), *Hibiscus* sp., *Parthenocissus quinquefolia* Planch. (Virginia creeper), *Prunus persica* Sieb. & Zucc. (peach seedlings), *Prunus salicina* Lindl. (Japanese plum), *Prunus serrulata* Lindl. var. *sachalinensis* Makino, *Pyrus malus* L. (apple), *Quercus prinus* L. (basket oak), *Rosa* sp. (rose), *Rubus* sp. (raspberry), *Rubus mesogaeus* Focke, *Salix acutifolia* Willd. (willow), *Tilia euchlora* Koch, *Tilia japonica* Simonkai (linden), *Ulmus americana* L. (American elm), *Vaccinium corymbosum* L. var. Adams (blueberry), *Vitis* sp. var. Concord (grape), *Zea mays* L. (corn), *Zinnia* sp.

It will be noted (Table II) that, with the exception of apple, slight or no injury resulted to the foliage of plants that had been sprayed with the combinations listed. The plants that were sprayed were growing under field conditions, a single application was made in each case by means of a hand sprayer, and the plants were examined for injury from three to six days later. The injury to apple foliage was probably due to the wetting agent, as Tergitol 7 penetrant (0.5 per cent) in water when applied alone to apple foliage caused severe burning, but did not injure the foliage of several other plants (Table II). Aside from this, no injury to plants resulted when ethylene glycol was used.

TABLE II  
DEGREE OF INJURY TO PLANTS BY ORGANIC CONTACT SPRAYS

Name of plant	Pyrethrum* resin 0.1% Acetone 1.0% Tergitol 7 pen- etrant** 0.5%	Methyl isobutyl ketone 1.0% Pyrethrum* resin 0.1% Tergitol 7 pen- etrant** 0.5%	Pyrethrum* resin 0.1% Tergitol 7 pen- etrant** 0.5%	Ethylene glycol 2.0% Tergitol 7 pen- etrant** 0.5%
Apple	xxx†	xxx	xx	x
Birch, white	—	—	o	—
Blueberry	—	—	o	—
Corn	—	—	o	—
<i>Corylus</i>	—	—	o	—
<i>Dahlia</i>	o	o	—	—
Elm	o	o	o	o
English ivy	o	x	o	—
<i>Gladiolus</i> sp. vars.	—	—	—	—
Purple Glory	—	—	o	—
Senorita	—	—	o	—
Grape	o	—	o	—
<i>Hibiscus</i>	—	—	o	—
Hollyhock	o	x	—	—
Horse-chestnut	—	—	o	—
Oak, basket	—	—	o	—
Peach	o	o	—	—
<i>Prunus salicina</i>	—	—	o	o
<i>Prunus serrulata</i>	—	—	o	—
Raspberry	o	o	—	—
Rose	o	o	o	o
<i>Rubus mesogaeus</i>	—	—	o	—
Snapdragon	x	o	o	o
<i>Tilia euchlora</i>	—	—	o	—
<i>Tilia japonica</i>	—	—	o	—
Virginia creeper	o	x	o	—
Willow	—	—	o	—
Zinnia	o	o	x	o

\* 0.02 per cent total pyrethrins.

\*\* 0.5 per cent Tergitol 7 penetrant alone caused injury only to apple foliage.

† o=no injury; x=slight injury; xx=moderate injury; xxx=severe injury.

#### METEOROLOGICAL FACTORS

Except for an excess of precipitation during July, 1938, the weather conditions prevailing during the time that the field experiments were con-

ducted were not unusual. According to the records of the nearest weather station (2, 3) the mean temperature for July was 76.6° F., which was only 0.18 degree above the previous ten-year July average. The mean temperature for August, 1938, was 78.2° F., exceeding by 3.47 degrees the ten-year average for that month. The precipitation for July, 1938, was 5.82 inches which was 1.6 inches above the average for the previous ten years, while that of August was 3.92 inches, which was 0.95 inch below the ten-year average. The maximum temperature reported during the period covered by the experiments mentioned above was 96° F., which occurred August 15, the minimum temperature for the same period was 60° F., which was recorded July 3.

The temperature during August, 1939, was unusually high with an average maximum of approximately 85° F., and an average minimum of approximately 70° F. The rainfall was light, between one and two inches.

#### SUMMARY

Preliminary tests were made of a number of organic compounds as possible contact insecticides for the control of adult Japanese beetle (*Popillia japonica*).

Tergitol 7 penetrant, a sulphated alcohol, can function both as a solvent and as a spreading agent for pyrethrum resins and possesses definite insecticidal properties of its own. It is an excellent spreading agent for pyrethrum sprays made up in acetone and methyl isobutyl ketone. Tergitol 7 penetrant at a concentration of 0.5 per cent gave a kill of adult Japanese beetle of about 50 per cent. An aqueous solution containing 0.02 per cent of total pyrethrins and 0.5 per cent Tergitol 7 penetrant gave a satisfactory control of the adults (85-100 per cent kill).

Plant tolerance to these sprays has been tested on 26 species of plants of which 21 species were tolerant, 1 species was severely injured, and 4 species only slightly injured.

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## VERTICAL MIGRATION OF JAPANESE BEETLE LARVAE

ALBERT HARTZELL AND GEORGE F. MCKENNA

Very little has been published on the movements of Japanese beetle larvae (*Popillia japonica* Newm.) in the soil. Chemical treatments of turf (5) are more effective if they can be applied at a time when the grubs are close to the surface and therefore a knowledge of the vertical migration of the grub population is important for the timely application of these treatments.

Hadley and Hawley (4) report a depth of from four to eight inches for the larvae. They state that movement ceases when the temperature goes below 50° F. Fox (1) gives 50° F. as the temperature below which movement ceases. He (2) believes that the larvae cannot stand a temperature below +15° F. (-9.4° C.). Friend (3, p. 644) has reported on the percentages of larvae of the Asiatic beetle (*Anomala orientalis* Waterhouse) found at different depths in the soil.

In order to study the vertical migration of the larvae of the Japanese beetle, sections of the turf of Gloucester loam soil type at the Boyce Thompson Arboretum were examined from time to time during the winter and spring of 1939. The investigation was directed principally toward the determination of the vertical migration of the grub population rather than a study of the up and down movement of the individual grubs. Evidence of lateral migration was observed also, although no exact determination was attempted.

Japanese beetle larvae made up between 77 per cent and 95 per cent of the total larval population in the plots studied; the remainder for the most part were *Autoserica castanea* Arr.

### METHODS

A method that was rapid had to be adopted due to the low soil temperatures found during the winter. A square of turf, which averaged about a foot to a side, was cut with a spade. The turf was removed and turned over so the soil was uppermost. Then the top soil was examined. Each two-inch layer of soil was kept separate. Usually two inches of soil was removed at a time. After the top two inches of soil was examined for grubs the next two inches of soil was taken out and examined. This continued until no grubs were found. In order to insure that no grubs were missed, usually six inches of soil was removed beyond the deepest larvae found. A record was kept of the grubs nearest the surface and of the deepest individuals. Soil temperatures were taken each time the turf was examined. Areas showing definite injury in the spring were sampled.

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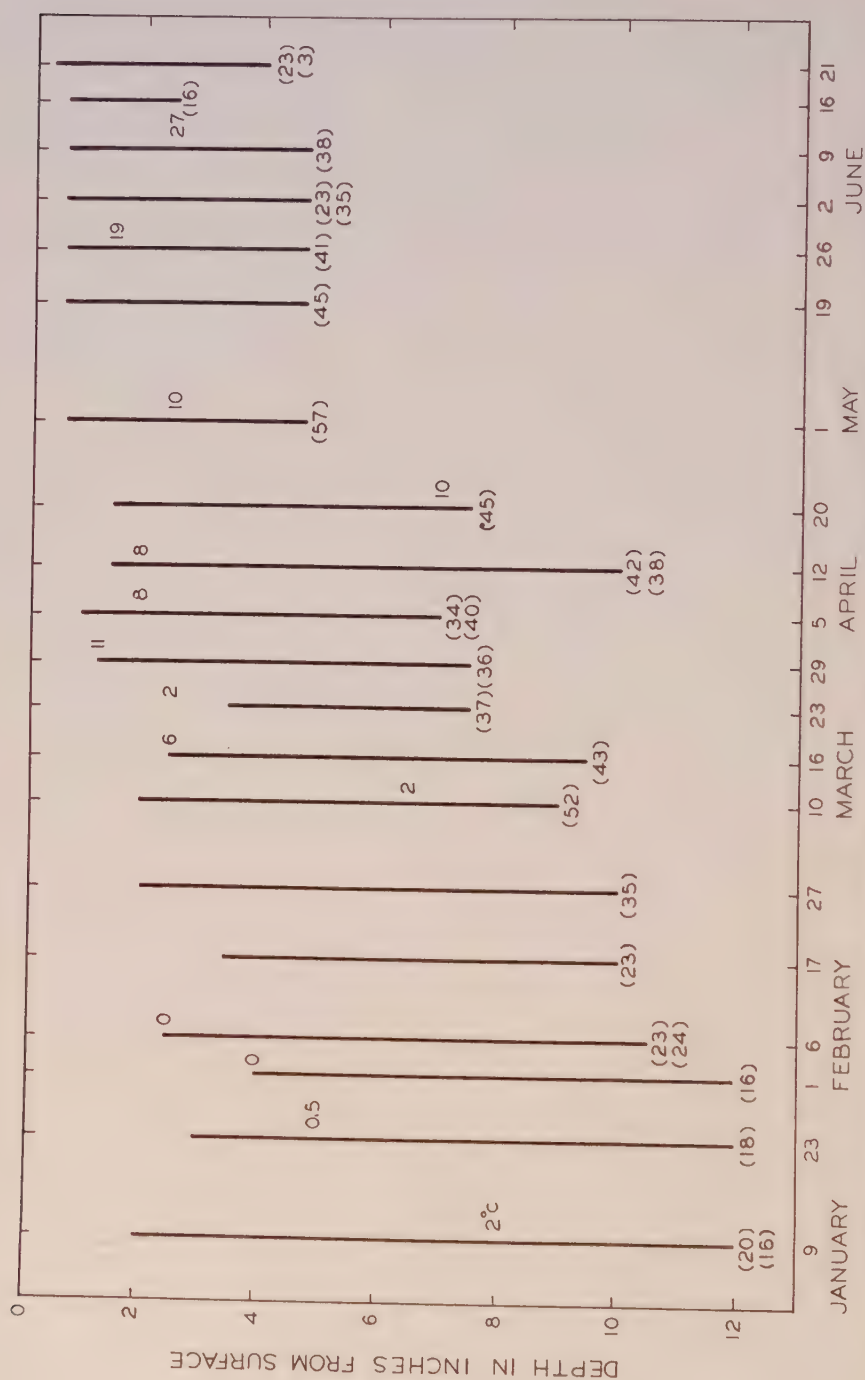


FIGURE 1. Diagram showing the range of depth of occurrence of Japanese beetle larvae. Figures at side of vertical lines indicate soil temperatures (°C.) at depth indicated and figures in parentheses total number of larvae found.

## RESULTS

The results are shown in Figures 1 and 2. On January 9 the first count was made. Larvae were found from 2 to 12 inches below the surface. The air temperature (5) in early January was not severe and very little snow fell. There never was any frost in the ground when the diggings were made. During the middle of January the daily air temperature decreased.

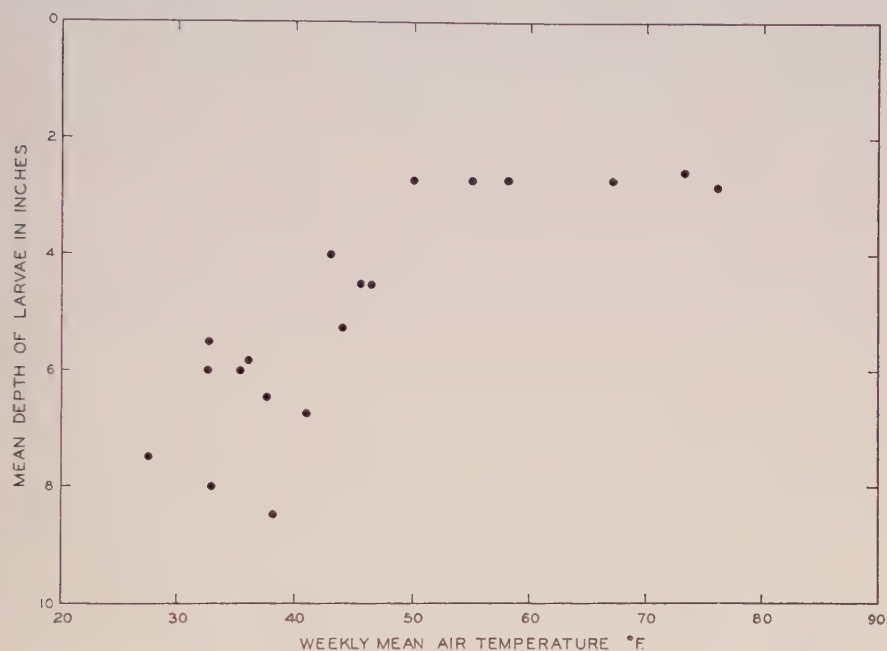


FIGURE 2. Correlation between air temperatures (°F.) and depth of larvae in soil.

February was the only month in which frost was found in the ground. Larvae were always found just below the frost line. They followed the frost line up and down. No larvae were found caught in the frozen area. During the last week of the month the air temperature rose and the vertical migration of grubs followed the rise in temperature.

During the early part of March the air temperatures dropped and the larvae descended. They came upward in the last part of the month due to warmer weather.

April was in general warmer than March and the larvae were up nearer the surface. The grubs were forced down in the middle of the month due to low air temperatures in the early part of the month. Warmer weather followed during the last of the month and the grubs were found nearer the surface.

During May and June the larvae were very close to the surface where they remained until they pupated. The maximum depth varied somewhat but was not as great as had been anticipated.

In early spring no greater difference in the grub population was observed under the injured turf than under good turf. Larvae were found nearer the surface in poor turf. Later in the spring the difference in numbers became more pronounced. In June only three larvae were found in soil where the grass had died. There seems to have been lateral migration in this instance as no dead grubs were found.

The maximum vertical movement observed was eleven and one-half inches. It is very doubtful if individual grubs moved that far. Probably the average movement for individual larvae was from four to seven inches. In general, upward and downward movement of grubs was from four to six days behind temperature fluctuations. There appears to be a definite correlation between the mean depth of the larvae and the mean air temperature (6), up to about 60° F., as illustrated in Figure 2. The variations on the same day are believed to be due to the location on the lawn and the amount of protective covering.

#### CLIMATIC FACTORS

According to the records of the nearest meteorological station (6), the 69-year (prior to 1937) mean for January was 31.5° F., 38.3° F. for March, 49.2° F. for April, 60.8° F. for May, and 70.0° F. for June. The mean for the corresponding months of 1939 was 32.2° F. for January, 39.2° F. for March, 48.2° F. for April, 64.9° F. for May, and 73.0° F. for June. The difference in all cases above is less than 5°. The mean for the corresponding months for 1937 and 1938 also did not exceed by more than 5°, except for January, 1937, which was 8.3° above the 69-year mean. February, 1939, was unusually warm, with a mean temperature of 37° F., which was 6.1° higher for that month than the 69-year average.

#### SUMMARY

Daily air temperatures influence the movement of Japanese beetle larvae in soil, but vertical migrations of the larvae were from four to six days behind temperature fluctuations. In general, the movement was only a few inches although the greatest vertical movement observed was eleven and one-half inches. The probable average movement for individual larvae was between four and seven inches. There appears to be a correlation between mean air temperature up to about 60° F. and mean depth of the larvae. Lateral movement seems to take place as the grubs move out of the injured areas to areas of good turf.

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## INTERMITTENT LIGHT AND THE FLOWERING OF GLADIOLUS AND CARNATION

JOHN M. ARTHUR AND EDWARD K. HARVILL

The present study is a continuation of the work with intermittent light reported last year (2). Many varieties of *Gladiolus* were included in the present study. These were chosen by Forman T. McLean mainly for size of both plant and flower of each major color type in order to increase the possible size of sweet scented hybrids produced by crosses with small sweet scented winter-flowering species (4). In order to give the control plants without supplementary light every possible advantage toward producing flowers, large corms three to three and one-fourth inches in diameter were obtained from a grower in Florida. These were kept in the cold room during the summer and planted on September 13 and October 13. In all, 162 pots of plants mainly with two shoots to each pot, including 25 varieties of gladioli, were grown under various types of lighting. All varieties flowered under one or more of the lighting conditions. All varieties and practically all plants of each variety flowered under intermittent lighting with 500-watt Mazda lamps. Seventeen pots of plants including 11 varieties failed to produce a single flower in the control house without supplementary light. The results on flowering with intermittent and continuous light and different types of lamps are compared.

Thirty-seven pots of carnations (*Dianthus caryophyllus* L.) were grown under intermittent light and 25 pots in the control greenhouse without supplementary light. From the lighted plants 204 flowers were cut to April 1 as compared with 30 from plants in the control greenhouse.

### LIGHTING

Five separate lighting circuits supplied different intermittent periods of lighting in a greenhouse of the ordinary type approximately 19 by 25 feet as follows. (A) One center bench 6.5 by 18 feet was illuminated by two rows of five each of the ordinary 500-watt Mazda lamps operated by means of a thermostat and electromagnetic switch located in the insulated greenhouse as described previously (2). (B) Two similar 500-watt lamps on the south side bench of this greenhouse were operated directly by a small soil-heating-cable thermostat suspended under one of the lamps (2). (C) Three 500-watt lamps on the same side bench were operated by a clock driven switch circuit which burned the lamps for approximately 8 minutes, 14 seconds, alternating with a dark period of 21 minutes, 45 seconds from 6:00 P.M. to 6:00 A.M. each night. (D) This circuit controlling three lamps on the north side bench was also operated by a clock switch which burned the

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TABLE I  
LIGHTING CIRCUITS, INTERMITTENCY, HOURS BURNED, AND CURRENT COSTS

Lighting circuit	A	B	C	D	E
Lamps operated	10—500-watt Mazda lamps, center bench	2—500-watt Mazda lamps, south side bench	3—500-watt Mazda lamps, south side bench	1—500-watt Mazda and 2—85-watt type H-3 cap. mercury lamps spaced at 55" and 18" respectively	2 sets of 2 tubes each, fluorescent 30-watt, 220-volt red or white spaced at 30"
Mechanism and switching frequency	Thermostat and magnetic switch in insulated greenhouse, 10-12 minutes on, 12-30 minutes off. On only at night and cloudy days.	Small soil-heating cable thermostat under one lamp, 3-9 minutes on, 3-30 minutes off, 6:00 p.m. to 6:00 a.m.	Clock mechanism, 8 $\frac{1}{4}$ minutes on, 21 $\frac{3}{4}$ minutes off, 6:00 p.m. to 6:00 a.m.	Clock mechanism, 1 hour, 33 min. on, 2 hours, 20 min. off, 6:00 p.m. to 6:00 a.m.	Continuous burn, 6:00 p.m. to 6:00 a.m. each night.
Average No. of hours per 24-hour day for month	Oct. Nov. Dec. Jan. Feb. March April	Started Oct. 10 4 hours, 17 minutes 3 hours, 59 minutes 4 hours, 41 minutes 4 hours, 27 minutes 5 hours, 15 minutes 5 hours, 28 minutes 4 hours, 38 minutes 3 hours, 30 minutes	Started Oct. 10 3 hours, 19 minutes to 3 hours, 39 minutes	Started Oct. 27 5 hours, 10 minutes 6 hours, 48 minutes 5 hours, 12 minutes 4 hours, 54 minutes 4 hours, 46 minutes 5 hours, 0 minutes	Started Oct. 27 12 hours, 0 minutes 12 hours, 0 minutes 12 hours, 0 minutes 12 hours, 0 minutes 12 hours, 0 minutes 12 hours, 0 minutes
Average for Dec. through April in hours and minutes per 24-hour day	4 hours, 8 minutes	4 hours, 53 minutes	3 hours, 29 minutes	5 hours, 18 minutes	12 hours, 0 minutes
Average current costs, cents per square yard per day at 2¢ per k.w.h.	3.17	4.07	2.91	4.46 for Mazda 1.74 for H-3	1.38 for 2 tubes spaced at 37-inch centers
A. Total cost dollars per sq. yd. from start to last of Jan.	\$2.65 384 W.	\$4.18 421 W.	\$3.60 421 W.	\$4.81 for Mazda 421 W. Mazda	\$1.34 57.8 W.
B. Watts per sq. yd. used	345 hrs.	497 hrs.	428 hrs.	\$1.87 for H-3 104 W. H-3	1164 hrs.
C. Total hours burned				571 hrs. H-3	



lamps for 1 hour, 33 minutes, alternating with a dark period of 2 hours, 10 minutes from 6:00 P.M. to 6:00 A.M. each night. The lamps consisted of a 500-watt Mazda lamp at one end of the bench and two 85-watt capillary mercury lamps,<sup>1</sup> type H-3, spaced 18 inches apart and at about 55 inches from the 500-watt lamp. (E) This circuit consisted of two 30-watt, 220-volt fluorescent tubes,<sup>1</sup> type "white" under a common plane-surface reflector and two similar tubes except "red" under a second reflector placed 30 inches farther along the bench. These lamps were turned on by a hand operated switch at 6:00 P.M. and burned continuously until 6:00 A.M. each night. The average number of hours and minutes per 24-hour day are shown in Table I, also the lighting circuits used. It will be noted that the number of hours of light in circuit A operated by the thermostat in the insulated greenhouse was abnormally high in March and April. This was due to the fact that after March 12, only eight out of the usual ten 500-watt lamps were used as a source of both heat and light in the insulated house. A ring of four sodium vapor lamps had been substituted for two of the Mazda lamps during the winter months. The sodium vapor lamps burned continuously each night until March 12 when they were turned off and only the eight lamps remained to heat the house. This resulted in an increase in the number of hours of light received on the center bench in the ordinary type of greenhouse lighted by circuit A.

The cost of current data given in the second from the bottom row of Table I are calculated for the average number of hours used per day given in the row above with lamps spaced as used in these tests. The center bench area (circuit A) contained 13 square yards while each of the side bench areas contained 8.3 square yards. Although only two H-3 lamps, and two sets of two tubes each of white and red fluorescent lamps were used, the wattage consumption per square yard is calculated at the rate of 16 H-3 or 16 sets of two tubes for a side bench containing 8.3 square yards. The effectiveness of the various lamps in lighting plants will be discussed in later paragraphs. It should be mentioned here that the fluorescent lamps as used were least effective while the type H-3 lamps were almost as effective as the Mazda lamps. The foot candle (f.c.) intensity at soil level (one foot from the lamp as measured by a photo-cell meter) for the Mazda circuits was approximately 750 and 200 f.c. at 32 inches. The white fluorescent tubes gave 75 f.c. at 16 inches from the tubes and 500 f.c. at one-half inch, while the red tubes gave only 15 f.c. at 18 inches and 50 f.c. at 3 inches. These intensities compare with 180 f.c. at 23 inches and 750 f.c. at 6 inches from the type H-3 lamps. The latter were enclosed in small spun-aluminum parabolic reflectors, while R.L.M. Ivanhoe porcelain reflectors were used with the 500-watt lamps. The fluorescent tubes were mounted in pairs on plane pieces of asbestos composition wall board 40 by 6.5 inches,

<sup>1</sup> These lamps were furnished by the General Electric Company.

which were coated with white enamel. The tubes were suspended so as to touch the tips of the growing plants, while the Mazda lamps were kept 12 to 18 inches above the tips. The H-3 lamps were also kept within 6 to 12 inches of the growing tips.

In the next to the bottom row of Table I, the costs in cents per square yard per average 24-hour day are given from December through April. In the bottom row of this table the total costs are given in dollars per square yard from October to the end of January. It is evident from these figures that the cheapest of the Mazda circuits is A which increases in the amount of light supplied as the average temperature decreases in the late fall and winter. Circuit C has the lowest daily current consumption, but owing to the constant number of hours supplied (3 hours, 29 minutes) reaches a higher total for the season. Circuit C could be easily changed to approximate both the effectiveness on plants and the total cost per square yard of circuit A by decreasing the hours per night burned in the late fall and late winter months. Without the use of a thermostat in circuit C, however, it becomes much less adaptable to accurate temperature control in the greenhouse and may easily result in overheating the tips of the plants with the result that less flowering is produced. In circuit D the H-3 lamps are most effective on plants and cheapest to operate, supplying a total of 571 hours for \$1.87. Circuit E with fluorescent lamps is least in cost, supplying 1164 hours for \$1.34 per square yard, but it is also least effective with plants. At least one more tube would be needed in each set to equal the effectiveness on plants of the type H-3 lamps. This would increase the cost by one-half to a total greater than the H-3 lamps but would supply twice as many hours of light in attaining the same production with plants. It is believed that no special advantage would be gained by operating fluorescent lamps on an intermittent schedule on account of their low energy output, while 500-watt Mazda lamps have shown a decided advantage operated intermittently, both in saving of current and in decreased injury to plants. The 500-watt lamp in circuit D was less effective than those in A, B, and C because of its relatively long period of burn (1 hour, 33 minutes, alternating with a dark period of 2 hours, 17 minutes) and consequent heat injury. This frequency was well suited, however, to the H-3 lamps in the D circuit. These lamps require several minutes to reach maximum brilliancy and after the circuit is opened will not come on again when the circuit is closed until a cooling period of several minutes has elapsed. On account of the high efficiency of the H-3 lamps (35 lumens per watt) and low heat output, plants can be exposed continuously to them so that the long period of burn in circuit D was favorable.

#### RESULTS WITH GLADIOLUS

Large size corms were obtained for the tests from a grower in Florida. These were kept in cold storage during the summer. Many of the corms

were three to three and one-fourth inches in diameter. It was felt that ability to flower might be more or less associated with bulb size especially in the control house without supplementary light. A few smaller corms of two or three varieties left over from the preceding winter were also used. The first planting of corms was made on September 13 and the second one month later. A single large corm was grown in each six-inch pot and the number of sprouts per corm was in every case limited to two of the most

TABLE II  
FLOWERING DATES OF VARIOUS VARIETIES OF GLADIOLUS

Variety	Planting date	In greenhouse with supplemental light		Approx. No. of days in summer sunlight to flowering*	Lighting circuit producing first flower
		Date of first flower	Approx. No. of days to flowering		
A. E. Amos	Oct. 13	Feb. 20	130	—	D
Ave Maria	Oct. 27	Feb. 24	120	89	C
Betty Nuttall	Sept. 13	Feb. 7	147	111	C
Chas. Dickens	Oct. 13	Feb. 7	117	93	B
Daintiness	Oct. 13	Feb. 7	117	—	A
Douglas	Sept. 13	Feb. 24	164	101	B
Douglas	Oct. 13	Mar. 2	140	—	A
Edith Robson	Oct. 27	Mar. 14	138	97	C
F. E. Bennett	Oct. 13	Feb. 18	128	98	D
Flaming Sword	Oct. 13	Mar. 2	140	91	A
Giant Nymph	Oct. 13	Feb. 18	128	89	D
Gold Eagle	Sept. 13	Dec. 25	103	80	B—C
Golden Dream	Oct. 13	Mar. 9	147	97	A
Greeley	Oct. 27	Mar. 2	136	87	D
Halley	Oct. 13	Feb. 7	117	81	B
Halley in constant light room } 400-w. H-1 and neon lamp }	Aug. 8	Oct. 31	84	81	—
Joergs White	Oct. 13	Feb. 7	117	97	A
Los Angeles	Sept. 13	Jan. 12	121	96	A
Minuet	Oct. 13	Mar. 2	140	97	A
Mammoth White	Sept. 13	Jan. 17	126	99	C
Pendleton	Sept. 13	Feb. 5	145	94	A—B—C
Picardy	Sept. 13	Jan. 24	133	92	A—C
Picardy in constant light room } 4 sodium vapor and inter- } mittent Mazda }	Sept. 23	Dec. 24	92	92	—
Red Gauntlet	Oct. 13	Feb. 8	118	—	C
Senorita	Oct. 13	Feb. 20	130	102	B
White Butterfly	Sept. 13	Dec. 18	96	86	A

\* Data supplied by Forman T. McLean.

vigorous. The varieties used were chosen by Forman T. McLean and grown for his work in cross-pollinating with small, sweet-scented varieties (5). They are listed in Table II. The dates of planting and appearance of first flower are also given, and for comparison the corresponding approximate number of days to flowering for the same varieties when planted in May in this region and grown out-of-doors in summer sunlight. The name of the variety will be found in the first vertical column while the planting

dates and flowering dates are in the second and third columns. It is realized that previous treatment of the corms as well as early spring temperatures are factors in determining the early growth rate of spring-planted bulbs and that this affects the number of days to flowering. Practically all of the bulbs grown under artificial light and planted either September 13 or October 13 had been stored for a sufficiently long period at 10° C. after a preliminary drying so that they were not dormant and therefore started growth immediately when planted.

The letters designating the electrical lighting circuit which produced the first flowers form the last column of Table II. The A and C circuits were most effective in producing the first flowers, B circuit was third, and D was fourth, while the fluorescent lamp circuits produced not a single first flower. Since the D circuit operated both a 500-watt Mazda and two H-3 capillary mercury lamps, it is not evident which of these was most effective. It should be stated that of the four first flowers produced under this circuit, the H-3 lamp was first in case of Giant Nymph and Miss Greeley and A. E. Amos flowered at the same time under both lamps while F. E. Bennett was first under the Mazda lamp.

#### INTERMITTENT AS COMPARED WITH CONTINUOUS MAZDA LIGHTING

A question often asked is: What advantage has intermittent lighting using higher wattage lamps over continuous lighting using lower wattage lamps? The extreme heating effects of 500-watt lamps can be partially avoided by using 100- or 200-watt lamps for longer periods and the difference in light intensity might in this manner be largely compensated by longer, continuous burning. The flowering data below supplied by F. E. Denny are included for comparison with the data already submitted for intermittent lighting. For this lighting, 200-watt Mazda lamps were used, spaced at 27.5-inch centers along a three-foot growing bench. The same type of R.L.M. reflectors were used as already outlined for the 500-watt lamps, and the plants were grown at the same time but in another greenhouse. The lamps were burned continuously for six hours each night and produced an intensity of about 180 f.c. at 38 inches, on the pots of soil in which the plants were growing. The corms were about the same size as the smaller ones used in the intermittent lighting tests and would have flowered under normal lighting conditions. The list of gladiolus plants exposed to the light with the number which flowered is as follows:

Alice Tiplady,	8	plants	exposed	to	light,	5	flowered
Giant Nymph,	8	"	"	"	"	, 1	"
Halley,	4	"	"	"	"	, 0	"
Laughing Water,	31	"	"	"	"	, 3	"
Picardy,	22	"	"	"	"	, 2	"



Souvenir,	43	plants	exposed	to	light,	10	flowered
White Butterfly,	49	"	"	"	"	, 21	"
Queen of Bremen,	16	"	"	"	"	, 10	"
Zona,	16	"	"	"	"	, 12	"

While not all of these same varieties were grown under intermittent light (five out of nine), it will be recalled that in all, 25 varieties of gladiolus were so grown and practically all plants of each variety flowered. It is believed that the failure of plants to flower under 200-watt continuously burning lamps is due mainly to the injury from heating rather than from the lower intensity of light supplied since plants flowered well under the 85-watt type H-3 mercury lamps at about this same order of intensity (circuit D, Table I). The heating effects of the H-3 lamps were, however, much less than the 200-watt Mazda lamps, both on account of the higher efficiency of the lamp (35 lumens per watt against 20 lumens per watt for Mazda) and shorter period of burning (on 1 hour, 33 minutes, alternated with off periods of 2 hours, 17 minutes). Another factor operating against flowering possibilities of plants under the 200-watt lamps was the higher night temperatures of about 65° F. as compared with 55° to 60° F. in the greenhouse with intermittent light. In all of the studies using supplementary light in greenhouses as well as with all artificial light in the constant light room, it has been observed that lamps of low efficiency (high infra-red output), such as the Mazda lamp, produce better growth and flowering at lower air temperatures while high efficiency lamps, such as the capillary mercury and fluorescent types, are generally still effective at much higher temperatures. That is, high ratios of infra-red to visible outputs of lamps are more favorable to plants at lower air temperatures. In a test made earlier with 500-watt Mazda lamps applied continuously for six hours each night within two feet of the tips of plants, gladiolus produced little or no flowering at 65° to 70° F. This failure to flower is doubtless a heat effect. In the present tests the plants flowered only fairly well under a 500-watt Mazda lamp in circuit D, Table I, alternating 1 hour, 33 minutes on, with 2 hours, 17 minutes off. Two plants of the Halley variety and two of Edith Robson failed to flower. Flowers of others were a little "soft" under this lamp—that is, failed to persist as long at the flowering stage. None of these undesirable effects was produced under 500-watt Mazda lamps in circuits A, B, and C where heat and light outputs were carefully regulated by either thermostats or short light period clocks. Here all plants flowered without exception and produced very fine, normal spikes of flowers. A photograph of several varieties of gladiolus grown with intermittent light is shown in Figure 1 A. The two small plants at the left are from the control house without additional light, none of which flowered. In these tests, just as in the previous year (2), plants under lights produced high yields of viable seed when properly pollinated.



FIGURE 1. A. *Gladiolus* plants flowering under intermittent light. The two plants at left are from the control greenhouse without light. B. Picardy and Giant Nymph *gladiolus*. Left, two plants grown in control greenhouse; right, two plants grown with intermittent light. C. Same plants as (B) showing new corms forming only on those plants in short day or control greenhouse which do not flower. D. Carnations on January 28. Left, control greenhouse; right, greenhouse with intermittent light.

The two entries in the flowering data of Table II under Halley and Picardy grown entirely without sunlight under two kinds of artificial light sources are of special interest. The pots of plants were exposed continuously to artificial light from the time of planting the corms in the case of Picardy and after the plants had grown a few inches above the soil in the case of Halley. The time required for development from the planting of the corm to the first flower under continuous light was the same as that of a summer crop in sunlight; 81 days for Halley and 92 for Picardy. Halley did not grow as vigorously under the capillary mercury and neon lamps as those in the greenhouse, although three plants out of four flowered. Three plants out of three Picardy exposed to the sodium vapor and Mazda lamps flowered and the plants grew as vigorously as those in the greenhouse, one flower spike attaining a height of 67 inches. The rate of development in this case was fully equal to June and July sunlight. The lighting in the case of Picardy consisted of four 10,000-lumen sodium vapor lamps arranged around a circle with an 18-inch porcelain reflector in the center carrying four 100-watt Mazda lamps. The sodium vapor lamps were burned continuously while the Mazda lamps burned intermittently as in (C) Table I. The air temperature was held continuously at 63° F.

#### NEW CORM PRODUCTION AND DAY LENGTH

In general, tuberization in plants has been more or less associated with short days. Garner (3) has discussed this briefly in a review of the work on photoperiodism, pointing out that onion is an exception in that bulb formation occurs on long days. Zimmerman and Hitchcock (6) found that capping the stem tips of artichokes from 4:30 P.M. to 9:00 A.M. produced tuberization and pointed out that the controlling influence is centered in the growing tip of the plant. In the present work with gladiolus it was observed that plants produced corms at once in the control greenhouse without supplementary light but such plants never flowered. In the lighted greenhouses new corms started to develop only after the flowers had opened and seed was setting. The only exceptions to this were a few plants in which the flowering stems were broken off. Such plants started to produce corms at once while under the long day influence. A few plants at the end of the center bench farthest away from the lamps failed to flower and produced corms early. This effect on Picardy and Giant Nymph varieties is illustrated in Figure 1 B and C. The photographs were taken on February 24 and show large new corms already grown on the two short day control plants (left) and no corm production but good flowering on the two lighted plants (right). The lighted plants later developed very large corms as the seed matured. The regulatory mechanism for corm formation in gladiolus is affected by flower and seed formation. The plants build new corms on either long or short days but flowering takes place only on long days and



development of the flower inhibits corm formation. Corm formation proceeds immediately following flowering. If the plants fail to flower for any reason, new corms are produced at once regardless of day length.

#### CARNATIONS AND INTERMITTENT LIGHT

The tests with carnations reported in the earlier publication (2) were continued for another growing season. In the earlier tests the plants were obtained late in the fall from a producer and were slow to start. In the present tests the plants were grown from cuttings taken in the preceding spring. Plants of the Dimity, Purity, and Crimson varieties were used. Thirty-one plants were grown under intermittent light (circuit A, Table I), and 25 under control conditions without light. In addition, a few plants were grown in the heat insulated house already described (2) and under circuit E with fluorescent tube lamps. The carnation plants in the insulated house were illuminated from 6:00 P.M. until 6:00 A.M. each night continuously by means of four 10,000-lumen sodium vapor lamps arranged in a ring formation as described in a previous publication (1). The first flowers were cut on December 18 from the insulated house, while the plants in circuits A and E (Table I) produced the first flowers on January 27. The first flower from the control plants was cut on February 10 but these plants did not produce effectively until the latter part of March. In all, 204 flowers were cut from 37 plants with lights in circuits A and E and insulated greenhouse to April 1, while only 30 were cut from the 25 controls without light. Sets of plants from the control and from the lighted greenhouse (circuit A) were photographed on January 28 and are shown as Figure 1 D.

The controls at the left were beginning to produce buds while many of the lighted plants at the right were already in flower. On May 9, the yield records were discontinued when 230 flowers had been cut from the lighted plants and 102 from the controls, averages of 6.2 and 4.8 flowers per plant respectively. It is evident therefore, that the lighted plants not only yielded flowers much earlier than controls but also the total crop yield was higher. Some flower stem weakness developed with progressive production under the Mazda lamps. This was in part due to the unfavorably high temperature (55° to 60° F.) and it is thought can largely be avoided by growing the plants at a lower temperature.

#### SUMMARY

1. Cost data on the supplementary lighting of greenhouses show that 500-watt Mazda lamps burning intermittently as operated by a thermostat are the cheapest and most effective source of light for plants at a total cost of about \$2.65 per square yard when operated to the last of January. Small 85-watt capillary mercury lamps as operated in these tests were almost as effective and cheaper in operating costs at \$1.87 per square yard.



The much greater initial cost of these lamps as compared with Mazdas would largely offset the difference in operating costs. Two 30-watt fluorescent tubes operated together as a single lighting unit were not as effective on plants even when burned each night continuously. The total operating cost of these tubes was \$1.34 per square yard. It is estimated that three tubes operated as a unit would be about as effective as the capillary mercury lamps.

2. Data for flowering on many varieties of gladiolus showed that practically all plants of every species were brought into flower under intermittent light from 500-watt Mazda lamps, while flowering was much more irregular under 200-watt Mazda lamps applied continuously for six hours each night.

3. New corm production went on under both short day and long day conditions but plants did not flower on short days. Plants under long day conditions flowered first and developed new corms only after flowers were opened. Where flower stalks were broken off, new corm formation took place at once under long day conditions, while new corms were formed at once under short day conditions without flowering.

4. Gladiolus plants were grown from corm to flower exposed continuously under all artificial light in about the same time as under sunlight during June and July.

5. Carnations were found to flower well under intermittent light thermostatically controlled. Two hundred and four flowers were cut from 37 plants with supplementary light, while only 30 were cut from 25 control plants without supplementary light.

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## STUDIES ON THE DEVELOPING COTTON FIBER.

### I. RELATION OF DEVELOPMENT OF CRUDE COTTON FIBER TO THE OTHER PRINCIPAL BOLL CONSTITUENTS<sup>1,2</sup>

JACK COMPTON AND FORREST E. HAVER, JR.

Considerable information has accumulated in recent years regarding the morphological and cytological characteristics of the developing cotton fiber (2-5, 7-12, 17, 18). Other than the contributions of Ivanova and Kurenova (16), Gallup (13), and of Caskey and Gallup (6) literature on the chemistry of the developing cotton fiber is practically non-existent. The purpose of the present investigation is to obtain more definite information as to the chemical composition and behavior of the many fiber constituents during growth as an aid in elucidating the structure of the mature cotton fiber. In the first communication of this series the relation of the crude cotton fiber to the other principal boll constituents during development will be considered for a pure bred American variety of cotton grown under two different environmental conditions, namely, in the field and in the greenhouse.

#### EXPERIMENTAL

##### SELECTION OF MATERIAL

The variety of cotton chosen for this investigation was Coker's Super Seven (*Gossypium hirsutum* L. Strain 4). The cotton plants were grown under two environmental conditions, namely, field and greenhouse. The former were grown at the South Carolina Experiment Station, Clemson College, South Carolina, during the summer of 1938, while the latter were grown (10) in the greenhouses of Boyce Thompson Institute during the winter and spring of 1938 and 1939.

In the case of field grown plants, flowers were marked with dated tags in such a manner that all the bolls reached the ages listed in Table I at the same time. In this way only one shipment was necessary for the whole series. By packing the bolls in moist paper and shipping by express, the bolls were received in good condition. In contrast to this procedure, flowers on the greenhouse plants were marked daily with dated tags and the bolls collected intermittently when the ages listed in Table II were reached. Sufficient flowers were tagged in either case so that well developed bolls could be selected. Despite careful selection certain anomalies usually arose during development, however, which could be neither predicted nor prevented that had a decided bearing on subsequent data.

<sup>1</sup> Presented before the Division of Cellulose Chemistry at the 98th Meeting of the American Chemical Society, Boston, Massachusetts, September 11, 1939.

<sup>2</sup> Cellulose Department, Chemical Foundation, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York.

TABLE I  
VARIATION OF BOLL CONSTITUENTS OF FIELD-GROWN COTTON DURING DEVELOPMENT (FALL, 1938)

Days after flowering	No. of bolls	Av. wt. of bolls, g.	Crude fiber				Fiber moisture in boll				Reducing sugars in fibers		Fats and waxes extracted from fiber				% seed + carpels
			Fiber + H <sub>2</sub> O + waxes + sugars		Dry fiber after H <sub>2</sub> O extraction		Fiber moisture in boll		Reducing sugars in fibers		Total		Residual, after H <sub>2</sub> O extraction				
			Wt., g.	% boll wt.	Wt., g.	% boll wt.	Wt., g.	% boll wt.	Wt., mg.	% boll wt.	Wt., mg.	% boll wt.	Wt., mg.	% boll wt.			
10	60	10.0	1.51	15.10	0.03	0.31	1.34	13.38	127.0	1.27	14.0	0.14	3.43	0.03	84.9		
15	29	21.8	4.41	20.20	0.15	0.70	4.06	18.64	102.4	0.75	30.7	0.14	6.58	0.03	79.5		
20	54	22.0	5.31	24.10	0.15	0.69	4.96	22.55	105.7	0.75	20.9	0.13	7.00	0.03	75.9		
25	55	24.5	5.04	20.60	0.89	3.62	3.99	16.30	111.1	0.45	45.8	0.19	10.84	0.04	70.5		
30	37	23.8	4.70	19.75	0.68	2.86	3.96	16.65	38.0	0.10	20.0	0.08	7.00	0.03	80.5		
35	27	23.3	5.11	21.90	0.87	3.74	4.18	17.95	24.0	0.10	28.7	0.12	6.20	0.03	78.2		
40	31	22.3	5.34	23.90	2.03	0.10	3.28	14.71	5.5	0.02	22.3	0.10	5.40	0.02	70.2		
45	23	21.7	5.18	23.87	1.00	8.76	3.26	15.02	7.0	0.03	13.0	0.06	4.30	0.02	70.1		
50	61	22.9	2.75	12.00	1.81	7.90	0.92	4.01	14.4	0.00	6.2	0.03	5.57	0.02	80.0		



## SEPARATION AND DETERMINATION OF BOLL CONSTITUENTS

After careful selection of the bolls, the calyces and bracts were removed by cutting smoothly with a sharp razor blade. The bolls were then wiped clean with a piece of cheesecloth and weighed (Tables I and II). Due to the difference in the manner of selecting the bolls of field cotton from that of the greenhouse cotton, slightly different procedures were used for determining the weights of the various boll constituents following dissection.

*Seeds and Carpels*

The bolls were opened by insertion of a scalpel along the lines where the carpels meet. The seed and fiber in immature bolls cling into a very compact mass which may be easily removed from the walls and partitions. The removal of the fibers from the seed offers considerable difficulty in very young bolls, 10- to 20-day, but may be easily accomplished in older material. The seeds delinted by hand still retained a small amount of very short fibers but this factor remains approximately constant and probably never exceeds 5 per cent of the total fiber weight. The dissected seeds and carpels were then immediately weighed. The higher moisture content of the cotton fibers obtained from greenhouse cotton causes the combined percentage yields of seeds and carpels to appear lower than that of the field cotton. On a moisture-free basis, however, the yields of these constituents from cotton grown under greenhouse and field conditions are not greatly different but in general are slightly higher when grown in the greenhouse.

*Crude Fiber*

*Wet crude fiber.* The weight of the fresh fibers was determined by subtracting the sums of the weights of seeds and carpels from the weights of the bolls. The fresh fiber mass thus obtained is a mixture of natural fiber moisture, sugars, fats and waxes, nitrogenous substances, cellulose, pectic materials, pentosans, and inorganic material (Tables I and II). Although it is the purpose of this investigation to determine each of the constituents of the fiber, the present report does not deal with all the constituents listed.

*Dry crude fiber.* The fresh fibrous material was placed directly into ice-water, thoroughly washed with water, and allowed to air-dry at room temperature. Moisture determination was then made on a portion of the air-dried material by heating at 105° C. in the usual manner and the weight of the total fiber mass corrected. The results are given in Tables I and II and Figure 1 A and B. This fibrous residue consists of fats and waxes, nitrogenous substances, cellulose, pectic materials, pentosans, and inorganic material.

TABLE II  
VARIATION OF BOLL CONSTITUENTS OF GREENHOUSE-GROWN COTTON DURING DEVELOPMENT (1938-1939)

Days after flowering	Wt. of boll, g.	Crude fiber				Fiber moisture in boll		Reducing sugars in fibers		Waxes extracted from dry fiber		Seed + carpels	
		Fiber + H <sub>2</sub> O + waxes + sugars		Dry fiber after H <sub>2</sub> O extraction		Wt., g.	% boll wt.	Wt., mg.	% boll wt.	Wt., mg.	% boll wt.	Wt., g.	% boll wt.
		Wt., g.	% boll wt.	Wt., g.	% boll wt.								
10	3.24	1.68	51.8	0.02	0.65	1.57	48.4	87.0	2.69	0.23	0.01	1.56	48.2
15	14.08	7.06	50.1	0.15	1.06	6.55	46.5	293.0	2.07	64.25	0.46	7.02	49.9
20	17.16	6.64	38.7	0.33	1.93	6.02	35.1	233.5	1.36	53.12	0.31	10.52	61.3
25	21.86	6.55	29.9	0.44	1.09	5.89	26.9	169.0	0.77	58.20	0.27	15.31	70.1
30	25.70	7.60	29.6	0.64	2.50	6.81	26.5	83.5	0.32	63.84	0.25	18.10	70.4
35	19.17	5.05	26.3	0.82	4.25	4.09	21.3	93.0	0.48	48.98	0.25	14.12	73.7
40	19.41	4.66	24.0	1.33	6.85	3.18	16.4	73.5	0.38	75.06	0.39	14.75	76.0
42	22.70	6.31	27.8	2.62	11.56	3.44	15.2	153.6	0.67	91.50	0.40	16.39	72.2
51	21.74	5.39	24.8	2.99	13.75	2.26	10.4	97.0	0.45	38.27	0.13	10.35	75.2
58	19.10	4.23	22.1	2.82	14.76	1.39	7.3	16.2	0.08	5.64	0.03	14.86	78.9

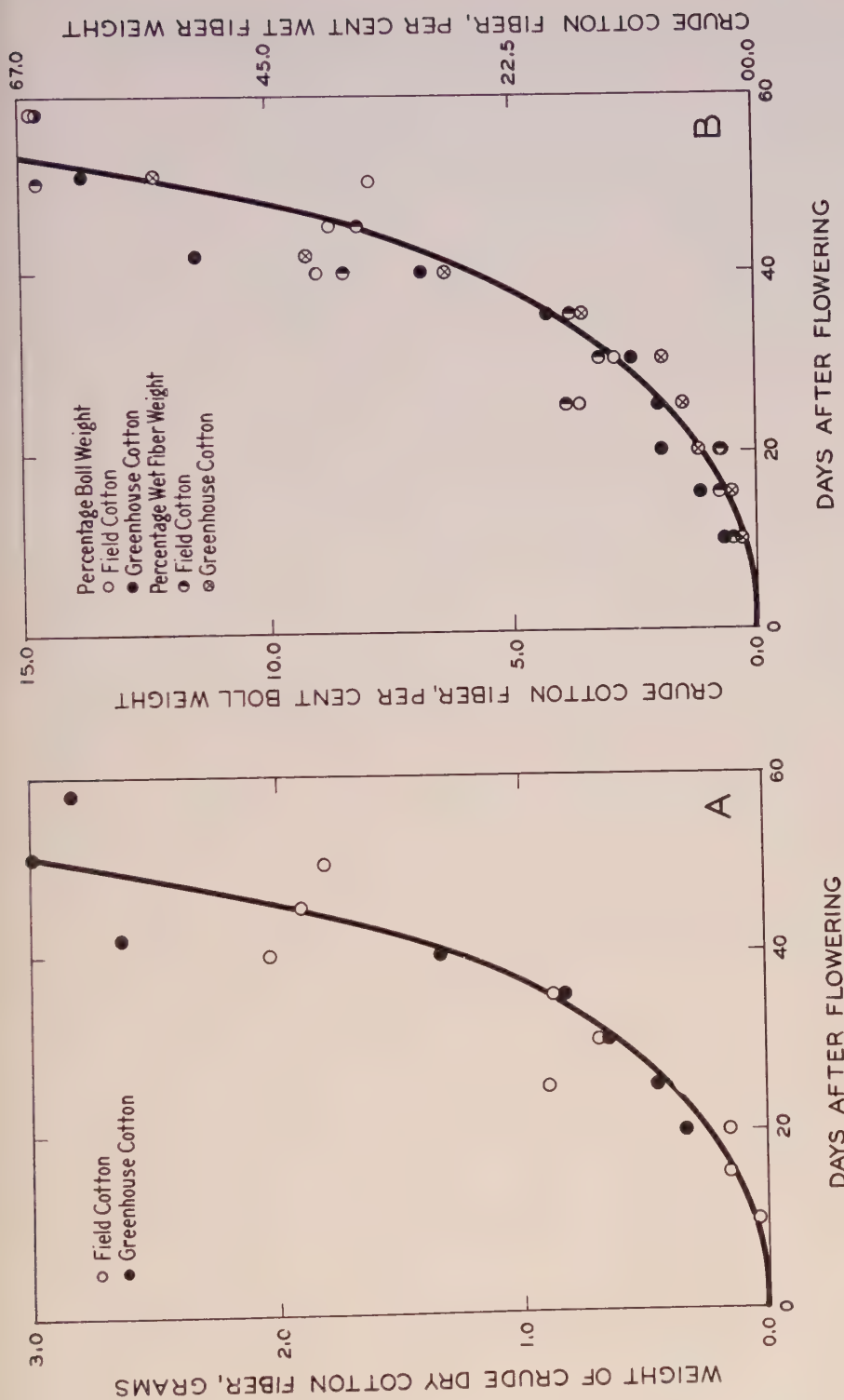


FIGURE 1. Rate of development of cotton fiber under different environmental conditions. A. Increase in weight of dry crude cotton fiber per boll with age. B. Percentage increase of dry crude cotton fiber with age based on the weight of the boll and on the weight of the wet crude cotton fiber.

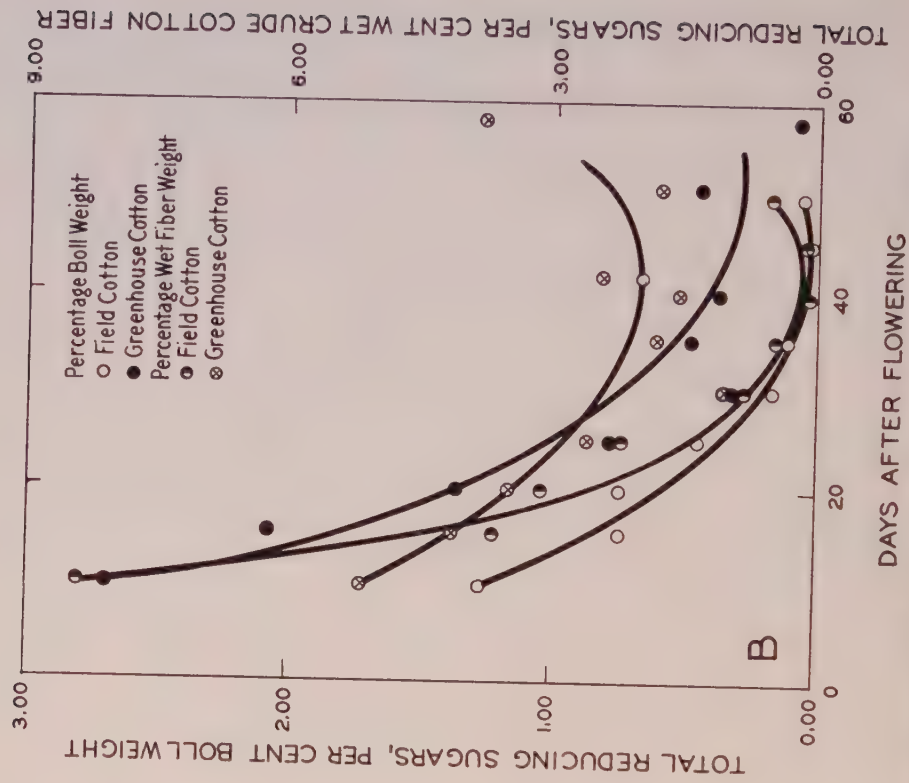
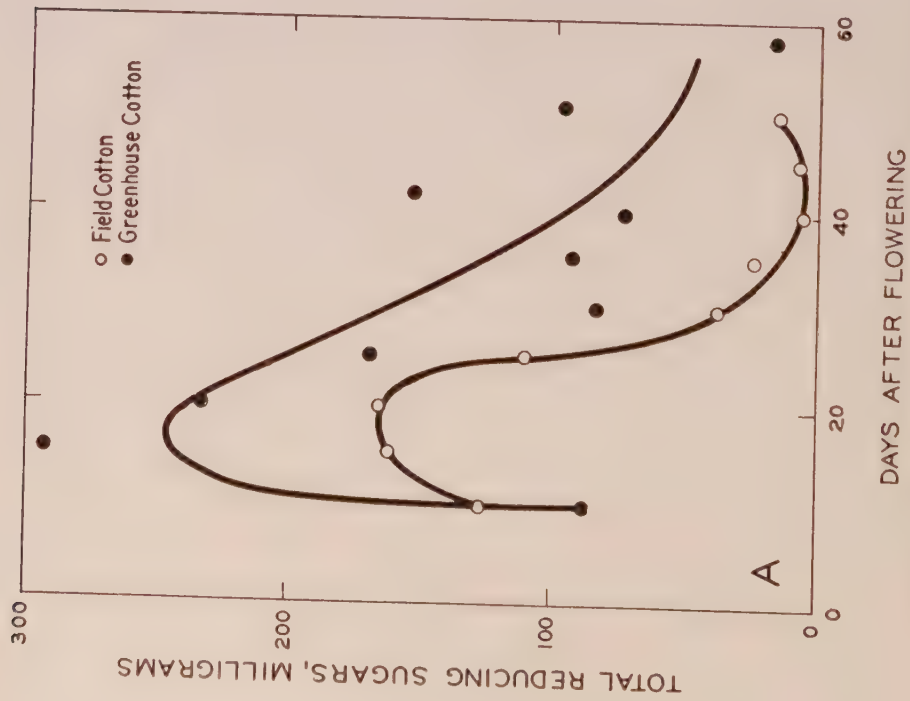


FIGURE 2. Variation of the total reducing sugars in the cotton fiber per boll during fiber development. A. Weight of total reducing sugars. B. Percentage of total reducing sugars based on the weight of the boll and on the weight of wet crude cotton fiber.



*Total Reducing Sugars in Developing Cotton Fibers*

The water extracts of the crude fibers obtained from field cotton bolls were diluted with twice their volume of 95 per cent ethyl alcohol and stored in tightly stoppered flasks at 0° C. until each could be analyzed. The precipitates, consisting largely of protoplasmic protein, were removed by filtration and the clear filtrates concentrated under diminished pressure at 40° C. to thick sirups. The protein-free sirups were then weighed and portions taken for analysis. Assuming the sirups to consist of 50 to 75 per cent reducing sugars, the portions weighed were usually such that when dissolved in water and diluted to 100 cc., solutions containing from 0.5 to 3 per cent sugar were obtained. Usually 1 to 3 cc. of this solution were then diluted to 100 cc. and the total reducing sugars determined on 10 cc. aliquots of the resulting solution by the Hanes modification (14) of the Hagedorn and Jensen method for total reducing sugars. Since the method only covers a range of total reducing sugars of from 0.5 mg. to 3.0 mg., it was often necessary to change the dilution ratio in such a manner as to conform with the analytical procedure. A blank was run each day during the analyses using 10 cc. of distilled water in place of the sugar solutions. For determining the actual sugar concentration from the observed titrations, the formula of Hulme and Narain (15),  $\text{sugar (mg.)} = b(T+a)$ , was used. In this formula  $T$  is the titration difference in cubic centimeters of 0.01 N sodium thiosulphate solution,  $a$  is a constant whose value is 0.05 cc. for all the sugars investigated, and  $b$ , a factor whose value depends upon the reducing power of the various sugars. In the present work the value of  $b$  was found to be equal to that of glucose and fructose, namely 0.340, which are the principal constituents of the sirups. Knowing the sugar content of the 10 cc. aliquot, the amounts and percentages of sugar in the sirups can be easily calculated. In a later report it will be shown that although pentoses are present in the sugar mixtures, the relative amounts are small compared with the combined amounts of glucose and fructose. The total reducing sugars occurring in the fibers during development as recorded in Table I and Figure 2 A and B are thus absolute values.

It was found expedient to use a slightly different procedure than that described above for determining the total reducing sugars in the fibers of bolls grown on cotton in the greenhouse. In this case, analyses were carried out on the entire sirups obtained from the fibers of single bolls. Thus the water extracts of the crude fiber were immediately concentrated under diminished pressure at 40° C. to thick sirups. The entire sirup of each extraction was dissolved in about 8 cc. of water, treated with 0.1 g. of calcium carbonate, heated in a boiling water bath for 5 minutes, and cooled in a manner similar to that described by Alten and Hille (1). After 30 minutes the mixture was again heated as previously described and then kept on ice for 18 to 24 hours. At the end of this time the heating was repeated as de-

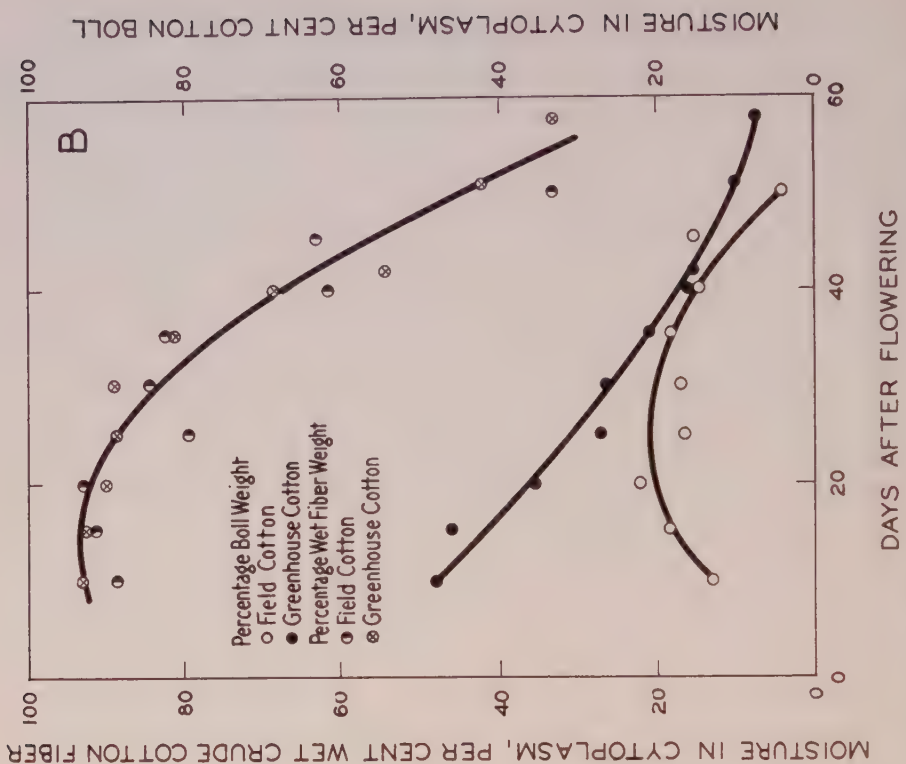
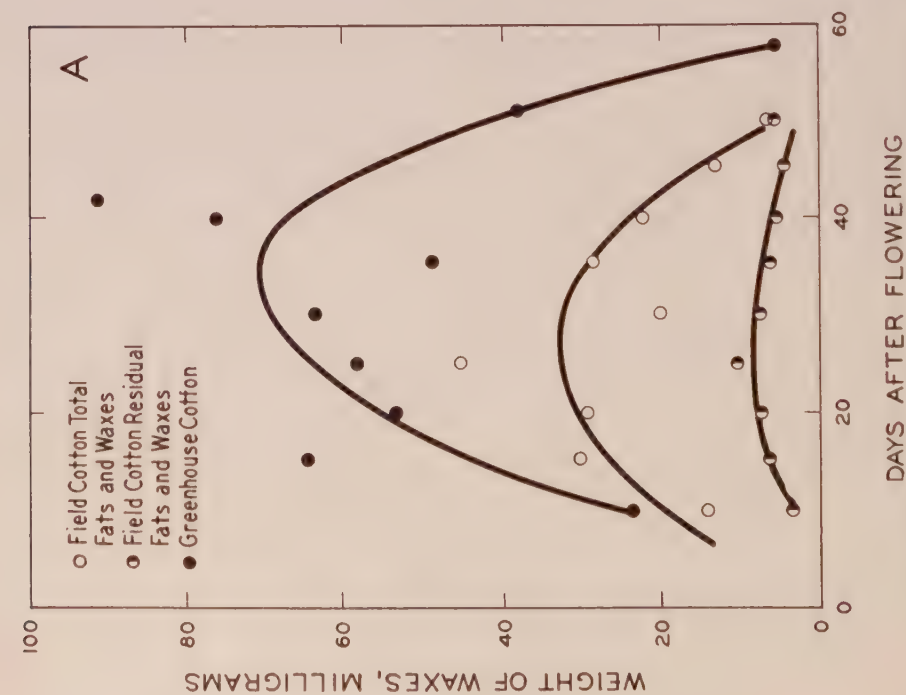


FIGURE 3. A. Variation in weight of the fat and wax constituent of the developing cotton fiber per boll as obtained by extraction with alcohol-benzene (1:1). B. Percentage moisture in the developing cotton fiber based on the weight of the cotton boll and on the weight of the wet crude cotton fiber.

scribed before. To the hot mixture there were then added 4 cc. of 96 per cent ethyl alcohol, the solution cooled, and filtered into a 25 cc. volumetric flask. The filter was washed three times with 4 cc. portions of 10 per cent ethyl alcohol and the filtrates diluted to the mark. According to the total reducing sugar, which was first determined by a rough approximation, from 1 to 4 cc. of this solution was diluted to 100 cc. and the total reducing sugars determined on a 10 cc. aliquot by the Hanes modification of the Hagedorn and Jensen method as previously described. The procedure described above for removing all substances other than sugars from plant juices (1) was also run on a sample of invert sugar. As there was no loss of reducing sugar by this process, the values given in Table II and Figure 2 A and B represent the total reducing sugars in the fibers.

After heating an aliquot of the sugar solutions obtained with 2 per cent  $\text{H}_2\text{SO}_4$  at  $70^\circ\text{C}$ . for 5 minutes, the total reducing sugars were again determined and found to be the same. This was taken as evidence that neither sucrose nor glycosides were present in the fiber extracts.

*Crude Fiber Fats and Waxes Soluble in Alcohol-Benzene (1:1)*

*Total crude fiber fats and waxes soluble in alcohol-benzene (1:1).* The crude fibers were removed from the bolls as previously described and dried as rapidly as possible at room temperature (18 to 24 hours). The weight of the crude dry fiber was then determined and the samples placed in a small Soxhlet apparatus. After extracting for 24 hours with alcohol-benzene (1:1), the extracts were concentrated to a gummy residue and weighed. The sugars which had been simultaneously extracted to a slight extent and mixed with the fats and waxes were determined by the Hanes modification of the Hagedorn and Jensen method. By difference the weights and percentages of the total fats and waxes extractable with alcohol-benzene were determined. From the average percentages found for each period of growth the approximate weights in the particular cases cited in Tables I and II and Figure 3 A can be calculated.

*Residual crude fiber fats and waxes soluble in alcohol-benzene (1:1).* In the procedure outlined above for the removal of the sugars from the fresh cotton fibers by washing in water, considerable quantities of the fats and waxes present in the protoplasm were lost. Thus the resulting dry fiber mass does not contain the total fats and waxes, but only that part remaining after this treatment. Nevertheless, it was of interest to determine the percentage of fats and waxes remaining which were soluble in alcohol-benzene (1:1). Accordingly, each of the washed and dried crude field cotton fiber samples was extracted in a Soxhlet apparatus for 24 hours with alcohol-benzene (1:1). The extracts were then concentrated under diminished pressure until removal of the solvent was complete and the residue weighed. The results are recorded in Table I. X-ray diffraction patterns

TABLE III  
PERCENTAGES AND RATION OF VARIOUS CONSTITUENTS OF FRESH CRUDE COTTON FIBERS WITHOUT PRELIMINARY DRYING

Days after flower- ing	% fiber moisture in boll		% fiber residue		% reducing sugar in fibers		% fiber waxes extracted alcohol- benzene (1:1)		Fiber:sugars:wax, ratio relative to fiber moisture of 100	
	Green- house cotton	Field cotton	Green- house cotton	Field cotton	Green- house cotton	Field cotton	Green- house cotton	Field cotton	Greenhouse cotton	Field cotton
10	93.4	88.6	1.25	2.05	5.18	8.41	0.14	0.93	1.3:5.5:0.2	2.3:9.5:1.0
15	92.8	92.1	2.12	3.47	4.15	3.68	0.91	0.69	2.3:4.5:1.0	3.8:4.0:0.7
20	90.6	93.4	5.00	2.88	3.51	3.12	0.80	0.56	5.5:3.9:0.9	3.1:3.3:0.6
25	89.6	79.2	6.67	17.61	2.58	2.20	0.89	0.91	7.4:2.9:1.0	22.2:2.7:1.1
30	89.7	84.3	8.46	14.47	1.09	0.81	0.84	0.43	9.4:1.2:0.9	17.1:1.0:0.5
35	81.0	81.8	16.13	17.10	1.84	0.47	0.97	0.56	20.0:2.3:1.2	20.0:0.6:0.7
40	68.2	61.4	28.55	38.01	1.57	0.10	1.03	0.42	41.8:2.3:2.4	61.9:0.2:0.7
42	54.5	—	41.58	—	2.43	—	1.45	—	76.3:4.5:2.7	—
45	—	62.9	—	36.67	—	0.13	—	0.25	—	58.2:0.2:0.4
50	—	33.4	—	65.82	—	0.52	—	0.23	—	197.0:1.6:0.7
51	41.9	—	55.47	—	1.80	—	0.71	—	132.5:4.3:1.7	—
58	33.1	—	66.66	—	3.83	—	0.13	—	201.4:11.6:0.4	—



taken of these samples show that the composition of this constituent changes slightly with age of the boll. The change is such as to indicate an increase in the length of long-chain fatty acid residues as the fiber matures.

### *Natural Moisture of the Developing Fiber in the Boll*

The weights and percentages of the moisture present in the fibers during growth can be obtained by subtracting the sums of the other wet crude fiber constituents from the weights and percentages of the wet crude fibers. Thus the sum of the weights of fiber fats and waxes plus reducing sugars plus dry crude fiber after water extraction, subtracted from the total weight of the wet dissected fibers, gives the approximate weight of the natural fiber moisture in the boll. These calculated values are given in Tables I, II, III, and Figure 3 B.

### RESULTS AND DISCUSSION

The thorough investigations of Ivanova and Kurenova (16) on the variation of cotton fiber constituents during growth were confined to a Russian variety of cotton, Naviotskii. Since the maturation period of the cotton fiber in this variety of cotton was about 80 days, the data are hardly applicable to American varieties of cotton. In the variety of cotton chosen for this work, Super Seven, the bolls matured on plants grown in the field in about 55 days, whereas the greenhouse material matured in about 60 to 65 days. Due to the difference in the maturation period of these varieties, it was necessary to repeat much of the work of these earlier investigators.

The gross constituents of the developing cotton bolls have been divided into two parts, namely, wet crude fibers, and seeds and carpels (Tables I and II). Inasmuch as the seeds and carpels have been reserved for future investigations, these fractions were discarded after obtaining the weight relation to wet crude fiber. The constituents of the wet crude fibers, on the other hand, were carefully studied.

The constituents of the wet crude fibers were divided into four groups: (1) dry crude fibers, (2) reducing sugars in the fibers, (3) fats and waxes extracted from the dry crude fibers with alcohol-benzene (1:1), and (4) natural fiber moisture. In Tables I and II the weights and percentages based on the weight of the bolls are recorded, whereas in Table III the percentages of each of these constituents are based on the weight of the wet crude fiber as obtained directly from the bolls. In Table III, the ratios of these fiber components are given at each stage of development.

In agreement with earlier observations (5, 7) the data in Tables I and II show that the weight of the cotton bolls reaches a maximum after about 25 to 30 days. During this time the wet crude fiber constituent of the bolls constitutes about 40 per cent of the total weight in the case of the greenhouse cotton, but only about 20 per cent in the case of field cotton. This is one of the outstanding differences in the bolls grown under these different

environmental conditions. The early difference in wet crude fiber percentage is soon overcome by the field-grown plants, and after the 25th day this difference is greatly diminished.

From the yields of dry crude fiber (Tables I, II) it will be observed that although the greater part of the early difference in wet crude fiber weight from the two sources is due to assimilated moisture, the primary fiber material actually develops more rapidly in the case of the greenhouse cotton. During the later periods of growth the rate of crude fiber development is about the same in either case. Before maturity is reached, however, the greenhouse cotton produces the greater percentage of crude fiber.

The importance of available moisture for the growing cotton plant upon the rate of fiber development has been pointed out by Balls (4), who was able to show a definite relation between periods of irrigation and length of lint. The bolls in which fiber development was going on at the time the water supply was most abundant made the longest lint. With the greenhouse cotton the moisture available to the plant was always high, whereas this condition did not exist in the case of the field cotton. This may explain the early differences noted in the two cases under consideration regarding the development of the crude fiber as well as the difference in late development.

The reducing sugars as probable precursors of the various fiber constituents are of considerable interest. In Tables I, II, III, and Figure 2 A and B the variations in total reducing sugars during fiber development are recorded. It will be noted that although the percentages of reducing sugars (Fig. 2 B) are not greatly different in greenhouse and field cotton bolls at the same periods of growth, the actual amount of sugars present in greenhouse cotton fibers is consistently greater (Fig. 2 A). Since the fiber constituents are synthesized over a longer period of time in the case of greenhouse cotton, a higher percentage of sugars should be present in the later stages of fiber development than with field cotton. That this is the case is shown in Table III and Figure 2 A and B. During the most active period of fiber development the ratios of the reducing sugars to the other fiber constituents of greenhouse and field cotton boll crude fiber are practically the same when the moisture content remains constant. These results in general conform with the observations of Gallup (13), Caskey and Gallup (6), and Ivanova and Kurenova (16) on the reducing sugars of cotton fibers at various stages of development. In contrast with the observations of the latter workers, however, no evidence has been found to indicate that reducing substances of considerably lower molecular weight than glucose are present in the developing fibers.

Although the wax-like outer limiting membrane is the chief constituent of the young developing cotton fiber, the percentage of fats and waxes that can be extracted with alcohol-benzene is relatively small (Tables I, II, III, and Fig. 3 A). It would appear, therefore, that if this primary substance is

composed to any considerable extent of fats and waxes, these substances are combined in such a manner as to be insoluble in organic solvents. In Table I and Figure 3 A, the effect of washing the fresh field cotton fibers at different ages with water on the resulting extractable fats and waxes is given. The difference in the two values approximates the protoplasmic fat and wax content of the developing fibers. At the time of maturity it will be observed that the residual fiber fats and waxes become practically the same as the total fats and waxes. During the final stages of dehiscence the total fats and waxes apparently become "fixed" in the fiber structure and can no longer be removed by simply washing in cold water. In Table III the ratios of the extractable fats and waxes to the other materials present in the developing fiber are given on the basis of a constant fiber moisture content. The numerical difference of these ratios approximates the uncombined fats and waxes which occur in the protoplasm of the cotton fiber.

Since the efficiency of the protoplasm in the formation of the various fiber constituents can be correlated directly with the available moisture, it was considered of interest to determine the change in the moisture content of the fiber during development. On the basis of the boll weight there is a considerable difference in the percentage of fiber moisture in the field cotton as compared with the greenhouse cotton (Tables I, II, and Fig. 3 B). The higher fiber moisture content of the latter probably accounts in part for the greater yields of the various fiber constituents obtained from bolls grown in the greenhouse. In Table III and Figure 3 B it will be seen that the percentage of moisture on the basis of the wet crude fibers, however, is practically the same in the developing bolls of field and greenhouse cotton at corresponding ages. As the fiber develops through deposition of cellulose and other membrane constituents the volume of the lumen diminishes and the total fiber moisture must also decrease. When the fiber moisture decreases to approximately one-third of the original amount fiber development apparently ceases.

In future communications the composition and characteristics of (a) the reducing sugars, (b) the fats and waxes soluble in organic solvents, and (c) the dry crude cotton fiber will be treated in detail. The ratios of the various fiber components given in Table III may then be better understood.

#### SUMMARY

A study has been made of the relationship existing between certain basic components of the developing cotton fiber. The components arbitrarily chosen were dry fiber residue, reducing sugars in the fiber, fats and waxes soluble in alcohol-benzene, and total fiber moisture. The variety of cotton selected was Coker's Super Seven (*Gossypium hirsutum* L. Strain 4) which was grown under both field and greenhouse conditions. Other than the slower rate of maturation and higher moisture content of the fibrous greenhouse material no essential difference seems to exist between the ratios of



the wet crude fiber constituents of bolls grown under field and greenhouse conditions. During the period of active fiber development the ratio, dry crude fiber:reducing sugars:waxes, on a constant-moisture basis, varies as follows: as the dry crude fiber mass increases the reducing sugars decrease, whereas the extractable fats and waxes remain practically constant.

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## A NEW RACE OF LILY HYBRIDS

A. C. McLEAN<sup>1</sup> AND FORMAN T. McLEAN

*Lilium tigrinum* Ker-Gawl. has had a reputation for sterility for many years. Dr. A. B. Stout and his associates have emphasized this, and explain it by the abnormal behavior of the triploid chromosomes at reduction division (1). He has also reported several attempts to hybridize this species with others (5), but succeeded only with the closely related species *maximowiczii* and *willmottiae*. Miss Preston has also used these successfully (4), but not other species crossed with *tigrinum*. Being interested in the disease tolerance of *L. tigrinum* and *L. ×umbellatum* and *L. ×elegans* hybrids, the authors undertook to intercross these two races of hybrid with *L. tigrinum* in 1934. We saved pollen of *L. ×umbellatum* and *L. ×elegans* hybrids, in cold storage from their normal time of bloom in June until the *L. tigrinum* bloomed in late July, and succeeded in making the cross. We used *L. tigrinum* pollen from plants forced early in the greenhouse on flowers of varieties of *×umbellatum* and *×elegans* growing in the garden, with equal success.

The plants of both reciprocal crosses flowered at our respective homes in late June and early July of 1937, and our resulting hybrids were substantially alike, being a new type of lily, approximately intermediate between the parent forms. All of them, comprising about a score of different seedlings, have the foliage and growth habits of the *L. umbellatum* group, with short stalks, usually three feet or less tall, and rather closely spaced spreading foliage, like *umbellatum*. But all of them bear brownish stem bulbils, resembling those of *L. tigrinum*. The flowers also are intermediate between the type of *L. tigrinum*, which is of the drooping, strongly recurved Martagon type, and the upright cup shape of flower of the *umbellatum* and *elegans* groups. They are wide open, neither cup-shaped nor strongly recurved, and are horizontal or nearly so, neither strongly drooping as in the Martagons nor upright. The colors cover much of the range of colors of the different varieties of *umbellatum* and *elegans*, being predominantly orange-red, shading in some to bright red, and in others toward buff yellow. All have large purplish-brown dots like those of *L. tigrinum*.

The general shape and posture of the blooms (Fig. 1) resembles that of Skinner's hybrid *Lilium ×scottiae*, which is a cross between an upright *umbellatum*-type lily and *L. willmottiae*, but these new *umbellatum-tigrinum* hybrids have the strongly spotted flowers of *L. tigrinum*, its bulbil-bearing habit, and a taller, more vigorous growth and wider range of color than has *scottiae*.

<sup>1</sup> County Agricultural Agent, Mercer County, Trenton, New Jersey.

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FIGURE 1. *Lilium* × *umbtig* 1 has the growth habits of its parent, *L. tigrinum* and *L. × umbellatum sapho*, and is orange-red and brown spotted.

To the best of our knowledge, this is the first time this particular cross with *L. tigrinum* has been made, and it would have been difficult without the pollen storage methods recently developed (2, 3).

The bulbils from the first of these to bloom have been grown to flowering size, and have proved to be thrifty and precocious, blooming the second year from the bulbils. Some of the old bulbs have been attacked by basal rot, but only one has shown mosaic symptoms, though several of them have been exposed to this disease at various times during the past five years. These hybrids are also fertile and set seed freely.

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## A LOOP METHOD OF DWARFING PLANTS AND INDUCING FLOWERING

FORMAN T. McLEAN

Many different treatments, both mechanical and chemical, have been used to dwarf trees and induce flowering and fruiting. The belief is quite general that the mere checking of vegetative growth—by root pruning, by drying, by girdling, etc.—will induce flowering and the setting of fruits, and this belief is borne out by experience in many instances. The simple method here reported was devised as a result of observations in Japan, in California, and elsewhere, but in its essential features it is new, to the best of the author's knowledge.

To check growth, the slender young stem of a sapling is bent into a loop with the tip pointing upward, the base and top ends of the loop are intertwined into a loose knot to hold the loop in place, and the young sapling is allowed to grow otherwise normally. As a result of this treatment, the elongation of the terminal shoot is checked, and growth is dwarfed. In the case of a two-year-old sweet cherry (*Prunus avium* L.) thus treated, flower buds were set the next year; the loop being made in the spring of 1938, the tree flowered in 1939. An untreated tree beside it has continued rapid terminal growth and not flowered, while the looped tree has formed laterals and fruit buds like an old tree, and ceased rapid terminal growth. Suckers formed below the loop have, of course, been pruned away, but the tree has not been otherwise trained or pruned. This is not an isolated case. Similar treatment of hemlocks (*Tsuga canadensis* Carr.), wisteria (*Wisteria sinensis* Sweet), chestnut (*Castanea dentata* Borkh.), and apple (*Pyrus malus* L.) induced similar checking of growth, but has not been continued long enough to induce early flowering of these plants (Fig. 1).

If this idea of dwarfing plants by mechanical means were at all new, then its application to only five such dissimilar plants as those enumerated would be a very slender basis for discussion. But the idea is very old. The Japanese have been distorting the stems of a great variety of woody plants and thus dwarfing them in their gardens for centuries, and the Chinese probably taught them how to do it in still earlier times. So there was little of originality to the scheme when the California experimenters found that by bending down the branches and upright shoots of their young prune trees, so that their tips pointed toward the ground, the trees were checked in growth, and induced to fruit early and heavily. In both of these cases the terminal shoots were left in a different position from normal. Since many investigators have found that the position and behavior of the terminal shoot has a strong influence on the activities of many parts of the

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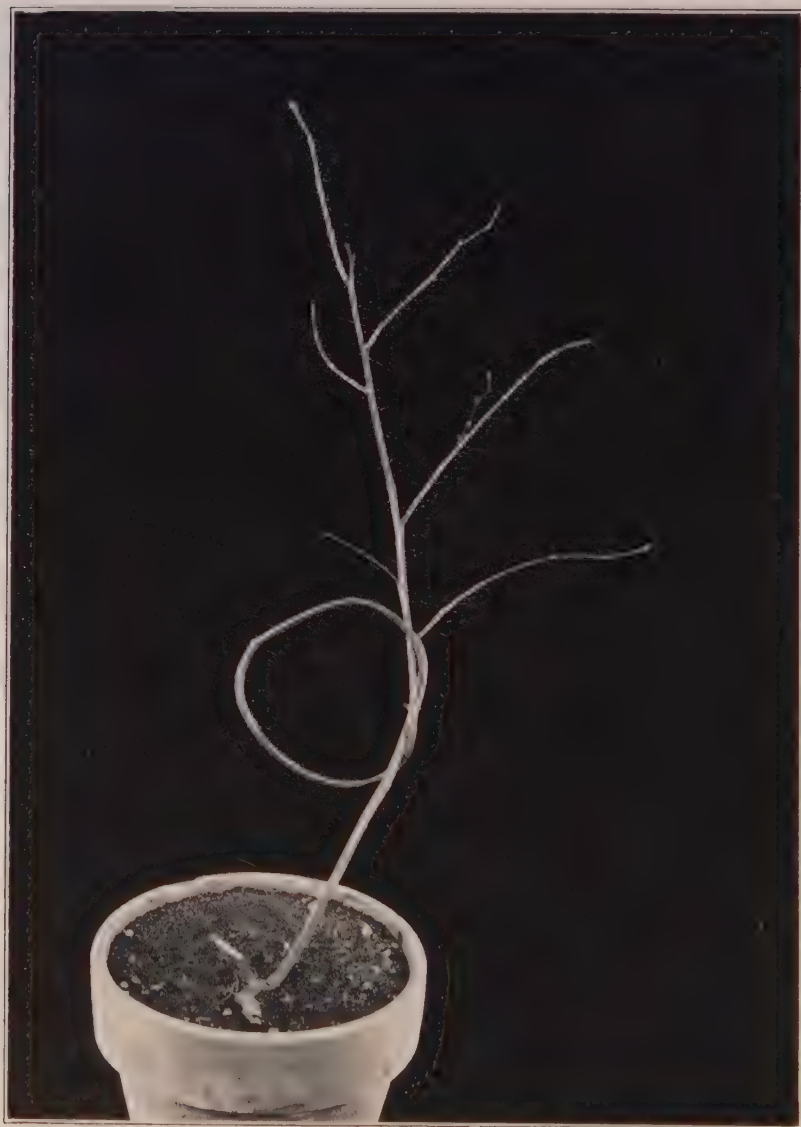


FIGURE 1. A yearling chestnut sprout dwarfed by tying the stem into a loop early the previous season.

plant, this might account for some of the growth check observed. But in the case of the looping, similar checking of growth was regularly observed when the terminal shoot and upper side branches were all left in normal positions.

The response of the wisteria, which is normally a twiner, is rather surprising. Though its stem is often twisted and distorted in its natural growth, two of three plants that were looped, one of them with a single loop and the other double, are dwarfed and shrubby in appearance. A third, in which the loop is half buried and thus allowed to root along the lower half of the circle, has sent out a vigorous, twining shoot, and has thus behaved quite normally for the species. What, then, is the cause of the dwarfing resulting from the twisting of the main stems of so many kinds of plants? Whether the stem be kinked or bent or looped, and whether the terminal shoot be left upright, horizontal, or upside down, the result is a dwarfing of the plant above the point of disturbance, often accompanied by early flowering and fruiting. Is the cause primarily mechanical interference with conduction?

The simple observed fact is that the looping of the woody stems of young trees, shrubs, or vines checks their vegetative growth and induces flowering without otherwise seriously impeding normal metabolic activities such as root or stem pruning or girdling or wounding would do. Accordingly, this may have practical utility in nursery and garden practice, for inducing early flowering of wisterias, etc., and may also have experimental value in the study of the causes and control of flowering of plants.





# ADVENTITIOUS SHOOTS AND ROOTS INDUCED BY NATURAL INFLUENCES AND SYNTHETIC GROWTH SUBSTANCES

P. W. ZIMMERMAN AND A. E. HITCHCOCK

The modern hormone theory assumes the control of growth and maturation by natural substances produced in various parts of the plant. Some kinds or phases of growth appear to fall in line with the theory while for others it is difficult of application. The induction of adventitious organs appears to fit best the claims of the theory. For example, a piece of root may grow buds at the proximal end and roots at the distal end, illustrating what is known as polarity. The influence causing this peculiarity appears to be of a chemical nature, since it can be interrupted by a discontinuity brought about by a notch or a piece of mica inserted in the stem. The theory is further supported by hormone-like responses induced with synthetic growth substances. Some fifty substances are known to have the capacity to regulate growth or have a formative effect in a way that differs from the effects brought about by organic foods or fertilizers (18). Strangely enough most of these compounds have the capacity to induce roots rather than other organs. No natural bud-regulating hormones have been chemically identified nor synthetic shoot-inducing substances yet discovered. There are, however, many observations and results of experiments which suggest that shoot-inducing and regulating substances do exist naturally in plants.

The purpose of this paper is to report the results of recent experiments involving adventitious shoots and roots (19).

## MATERIALS AND METHODS

The principal material used in the experiments consisted of shoot and root cuttings of the following species: *Actinidia arguta* Miq., *Amelosorbus jackii* Rheder, *Elaeagnus umbellata* Thunb., *Hibiscus syriacus* L. (althea, Rose of Sharon), *Linum usitatissimum* L. (flax), *Populus generosa* Henry (hybrid), *Populus* sp., *Pyrus malus* L. var. *niedwetzkyana* Aschers. & Graebn. (Purple crab apple), and *Syringa vulgaris* L. (French lilac). A few experiments involved root cuttings of *Paulownia tomentosa* Steud. and leaf cuttings of *Saintpaulia ionantha* Wendl. (African violet).

Stem cuttings four to six inches in length were prepared by removing some or all of the natural buds. After special treatments consisting of bark removal, exposure to vapors of growth substances or ultra-violet light, the cuttings or separated parts were placed in moist chambers provided by means of bell jars, inverted beakers, Petri dishes, or stone jars containing moist peat moss. When under bell jars the cuttings were placed upright in

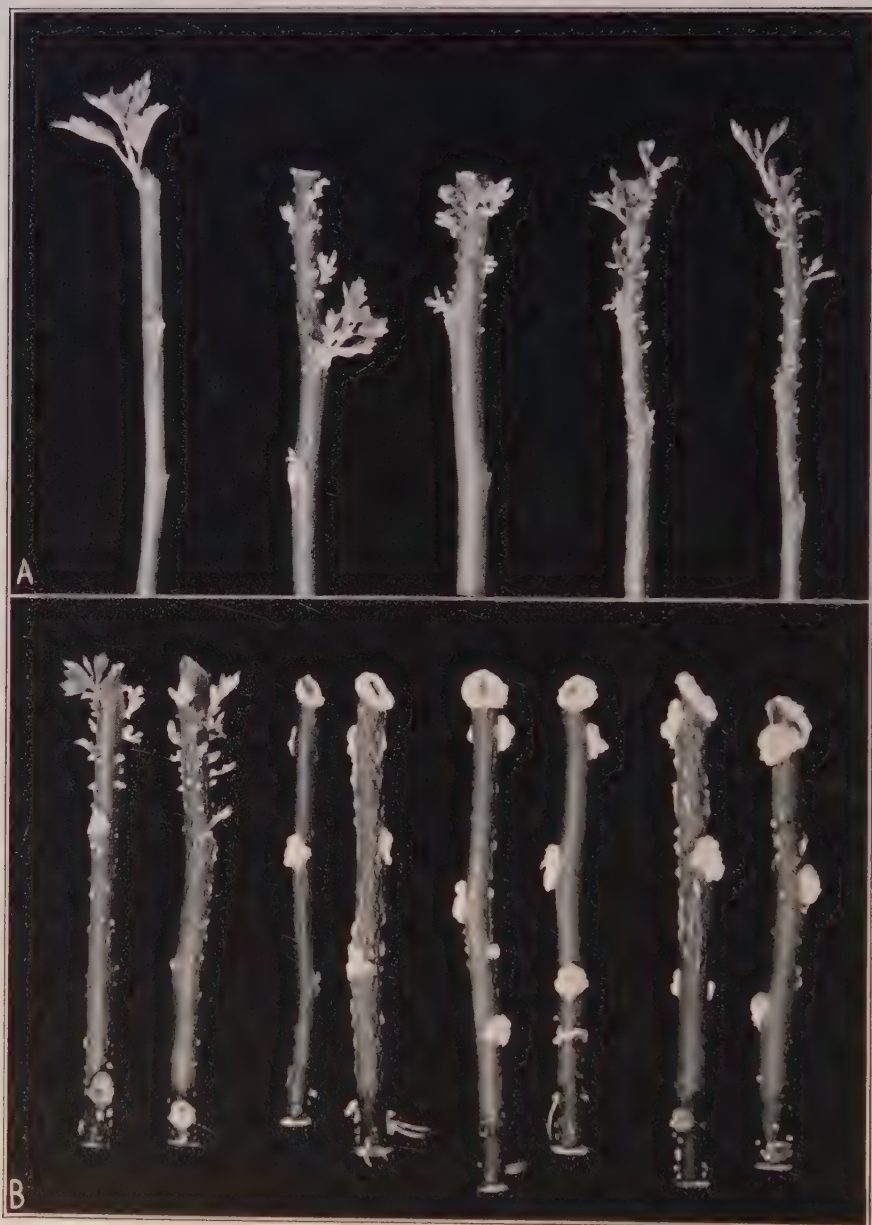


FIGURE 1. *Althea* cutting showing adventitious shoots and roots and induced callus. A. Left, one cutting with shoot from normal bud at top. Right, four disbudded cuttings showing adventitious shoots after 21 days. B. Disbudded cuttings in pairs. Left to right: controls, exposed to vapors of methyl indolebutyrate, exposed to vapors of methyl indoleacetate, exposed to vapors of methyl naphthaleneacetate.

beakers with approximately one-half inch of water. Moist peat moss was placed loosely over upright cuttings in stone jars. In Petri dishes the cuttings were placed on moist filter paper. To treat with vapors of growth substances the cuttings were placed upright in beakers and exposed for a few minutes under bell jars or beakers to vapors produced by warming the equivalent of a drop of the substance in a watch glass on a hot crucible. The esters of the various acids were most readily volatilized (18). The phenyl esters required little or no heat. The most satisfactory methods for storing cuttings upright in light was an inverted 1000-cc. Pyrex beaker over, but not sealed on, a glass plate. The Petri dish with moist filter paper was satisfactory for horizontal cuttings. If the containers were closed too tightly the cuttings became contaminated with microorganisms. The ultra-violet light prevented to some extent the attack by bacteria and fungi. The cuttings were covered with glass that transmitted much of the ultra-violet light. Pyrex glass was found satisfactory.

#### RESULTS

*Hibiscus syriacus*. Control cuttings with buds intact produced shoots from the uppermost two or three buds, showing the usual polarity. When the uppermost bud was removed, adventitious shoots appeared near the top of the cutting but not at the lower end. When all the buds were removed adventitious shoots appeared from approximately the upper half of the cuttings, varying with the age of the wood and the condition of the tissue. This response is illustrated in Figure 1 A. The new shoots came from the callus at the scars where buds had been removed, from the apical cut end, as well as from the bark. The first evidence of growth from the bark appeared like proliferating lenticels. Soon thereafter the epidermal hairs of the first leaves could be seen with the aid of a lens. The shoots and buds could be seen with the naked eye 8 to 15 days after the experiment was started. All the new buds appeared to grow at an equal rate for a few days but soon the uppermost shoots grew most rapidly and inhibited those below. Those on opposite sides near the apical end did not appear to influence each other. Also, occasionally a shoot arising from the callus of a scar grew at a normal rate though some distance down the stem from other active shoots. Inhibited shoots started growth again when a portion of the stem above was removed. Here again the uppermost active shoots dominated those lower down the stem. Cuttings of the current season frequently produced only few adventitious buds near the apical end. Cuttings of two-year-old wood produced from 50 to 100 adventitious buds often extending from the tip to near the basal end. The majority of initiated buds did not grow beyond the bud stage due, assumably, to the correlative influence of active shoots above. Shoot primordia could always be distinguished from those of roots by epidermal hairs which could be seen with a lens before





FIGURE 2. *Althea* cuttings, disbudded and otherwise mutilated to show tissues giving rise to adventitious shoots. A. Left, disbudded cuttings producing shoots and roots. Middle, wood with bark just removed. Right, wood with callus and adventitious buds 59 days after bark was removed. B. Bark and wood producing callus and adventitious shoots. Left, three cuttings with half of bark removed. Right, bark showing callus and beginnings of adventitious shoots from inner side and at edge.



the leaves were evident. Also, the buds developed chlorophyll quickly, whereas the roots remained white. There was often a kind of transition zone near the base of the cutting marking the end of the bud region and the beginning of roots. It appeared that the tissue in the vicinity of the lenticels had the potentialities for the initiation of either shoots or roots. The ones actually produced may have been determined by the dominating chemical influence.

To test this theory, disbudded cuttings were treated with vapors of root-inducing substances so that the entire stem would be under the influence of known compounds. These substances completely inhibited initiation of the adventitious shoots and instead root primordia formed throughout the entire length of the cutting. Thus the normal shoot-root polarity was entirely overcome. Figure 1 B shows controls producing shoots and three sets of cuttings producing roots after treatment with three of the most effective substances—the methyl esters of indolebutyric, indoleacetic, and naphthaleneacetic acids. The phenyl substances (methyl and ethyl phenylacetate and *cis* cinnamic acid) did not prevent shoots from forming unless the concentration was very high and the tissues were injured.

Root cuttings of althea formed numerous adventitious shoots from the cut surface of the proximal end. A few shoots came through the bark near the edge of the cut end. Roots came from the distal end. This indicates even stronger natural polarity than shown by stem cuttings.

On disbudded stem cuttings shoots push through the bark, arise from the callus growing where buds were removed, and also at the apical cut end of the stem. In order to determine more definitely the tissues giving rise to buds, the wood and bark were separated and kept in high humidity until new buds formed. Adventitious buds were initiated from both bark and wood as shown in Figure 2 A and B. Callus formed from pith over the surface of the wood and then shoots were initiated from end to end of the cutting. Roots were not produced and there was no apparent polarity effect as was shown where bark and wood remained together.

The bark produced callus on the inside, possibly due to cambial strands clinging to the surface. Most activity appeared at the edge where the cut was made in order to remove the bark from the wood. At this place the first signs of adventitious buds were observed. With a lens they appeared as small green spots with epidermal hairs. In a few cases buds protruded through the outer surface of removed bark as well as being formed on the callused inner side. When bark was removed from only one side of the stem, adventitious buds were initiated abundantly at the edge where the remaining bark and wood joined. In this case new shoots came also through the intact bark. The exposed wood formed callus and then produced adventitious buds. The callus was not uniform over the exposed wood, indicating that the cambium had been removed in places. Humps of callus formed at

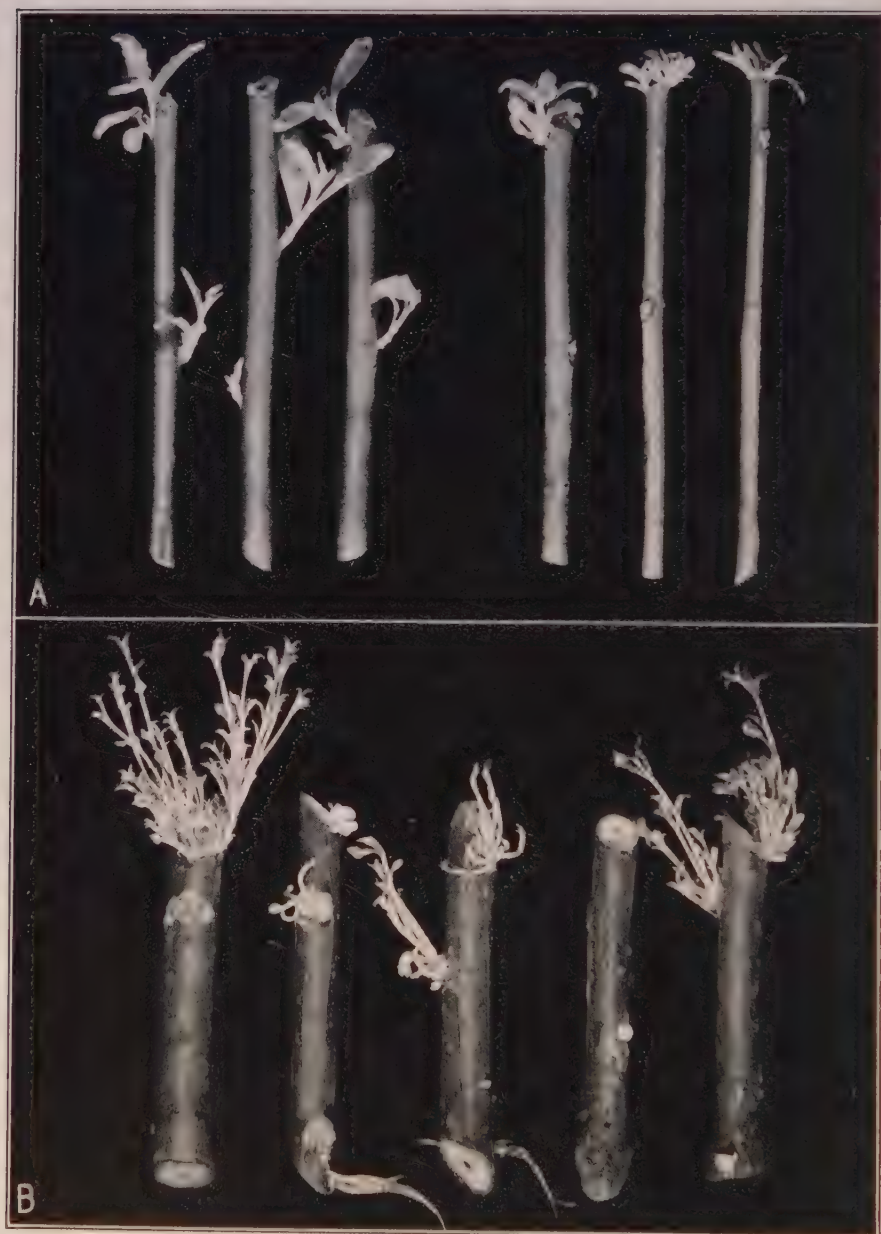


FIGURE 3. A. *Elaeagnus* cuttings. Left, with natural buds intact. Right, disbudded cuttings giving rise to adventitious shoots. B. *Populus generosa* cuttings after 18 days. Left to right: control; exposed to vapors of methyl indoleacetate, methyl indolebutyrate, methyl naphthaleneacetate, and methyl phenylacetate. The callus and adventitious buds had formed before exposure to the vapors. Note inhibition and distortion of shoots.

the pith or wherever xylem tissue extended above the average surface. It was observed that newly-stripped wood had many xylem points which had extended into the bark. Newly-formed adventitious buds were usually associated with these projections. Lenticels might also have been located over the projections but this would not account for all the lenticels or all the potential adventitious buds.

It was difficult to prevent contamination when the bark was removed from the wood unless ultra-violet light was used occasionally. It was effective when the Petri dishes were covered with glass transmitting ultra-violet light as well as when the cuttings were exposed directly. Direct exposure of more than one minute injured the newly-exposed tissue.

*Populus generosa*. Cuttings of poplars formed adventitious buds from callus at the apical end or along the stem where buds had been removed, as shown by Simon (15). No shoots came through the bark as occurred with the althea cuttings. There was some evidence of polarity in poplar cuttings but not as striking as with althea. However, many more shoots formed from callus at the apical end than from that at the base of the cutting. Figure 3 B shows the effect of growth substances applied after callus had formed on poplar cuttings. Methyl naphthaleneacetate completely inhibited bud development. The indole substances retarded and distorted shoot development. Phenyl substances did not retard shoot development. Cuttings treated at the time they were made formed large callus growths but no adventitious buds. *Populus* species vary greatly in their capacity to regenerate shoots, some producing adventitious buds abundantly while others not at all or sparingly.

*Elaeagnus umbellata*. Stem cuttings of *Elaeagnus* with buds removed produced adventitious buds at the apical end and also at the scars where buds were cut away (Fig. 3 A). No new shoots came through the bark. In this respect the response of *Elaeagnus* resembled *Populus* more than *Hibiscus*.

*Paulownia tomentosa*. Root cuttings of *Paulownia* produced adventitious shoots from the upper end only, but not at the cut surface. The new buds pushed through the bark, scattered sparsely over the upper inch. In contrast to *Paulownia*, *Hibiscus* root cuttings produced adventitious shoots largely from the cut surface.

*Linum usitatissimum*. Flax seedlings decapitated just below the cotyledon leaves regenerated from one to ten shoots from the hypocotyl stump, as often mentioned in the literature (4, 11). The number of adventitious shoots produced by flax could not be increased by treatment with root-inducing substances applied as solutions or vapors. When the dosage was sufficiently high to be effective, bud formation was inhibited.

*Saintpaulia ionantha*. Leaf cuttings of *Saintpaulia* produced adventitious buds at the basal part of a petiole or severed veins of the leaf blade.



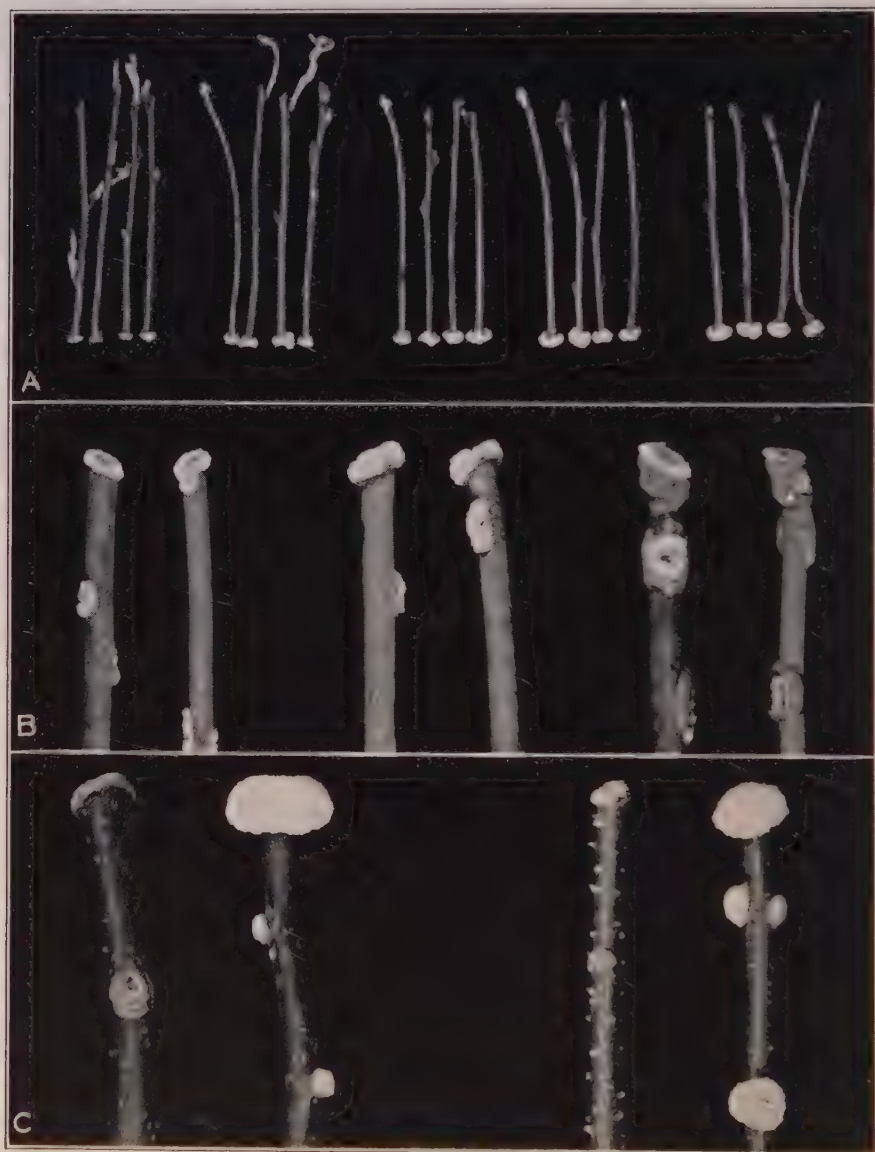


FIGURE 4. Induced callus. A. *Amelosorbus* cuttings treated for 24 hours with water solution of naphthaleneacetic acid. Left to right: control, 10 mg./l., 20 mg./l., 40 mg./l., 80 mg./l. (Photographed after 15 days.) B. *Actinidia*, disbudded cuttings, photographed 16 days after exposure to growth substance vapors. Left, control; middle, methyl indoleacetate; right, methyl naphthaleneacetate. C. Left, Purple crab. Control cutting and one exposed to vapors of methyl indoleacetate. Right, French lilac. Control and one exposed to vapors of methyl indoleacetate. (Photographed after 27 days.)



Strong polarity was evident. Treatment of the regenerating parts with indole or naphthalene root-inducing substances increased the rooting capacity of the tissue but reduced the number of buds and delayed their development.

*Callus induced.* Root-inducing substances are known to accelerate callus formation also. Figure 4 A shows an increase of callus at the base of *Amelosorbus* cuttings with increase in concentration of indolebutyric acid. Similar responses have been previously mentioned.

When the cuttings were exposed to vapors of growth substances, certain qualitative differences were evident from the callus formed and the roots induced. Figures 1 B and 4 B illustrate this fact. The naphthalene ester caused callus and swelling in the bark of *Actinidia* some distance back from the cut surface. Methyl indoleacetate induced an abundance of callus at the cut surface without swelling of the bark. Methyl indolebutyrate caused the least callus but induced roots. In general, vapors of methyl indoleacetate caused the largest callus formation, resembling tumors or galls as illustrated with Purple crab and French lilac in Figure 4 C.

When in light the callus of control cuttings developed enough chlorophyll to cause a green color. In contrast to this, callus induced with the most effective growth substances remained white, as did roots which developed with or without chemical treatment. This suggests some relationship between root tissue and callus stimulated with root-inducing substances.

*Light.* Light was not a controlling factor for the formation of adventitious buds on mature stem tissue. *Althea* cuttings in the dark formed shoots as readily as cuttings in the light. For development of newly-formed shoots, light was of course necessary.

*Effect of air and gas mixtures.* Several interesting variations were induced by varying the air and gas mixtures during the first few days after the cuttings were made. For example, a high carbon dioxide and low oxygen content for five days caused more adventitious buds to form on *althea* cuttings than high oxygen and low carbon dioxide (Fig. 5 A). Also, ethylene (1 part to 100,000 parts of air) in the air for six days caused an increase in the number of buds formed. Cuttings treated with high (near 100 per cent) concentrations of oxygen and nitrogen responded much like controls in air. Experiments involving various proportions of different gases in the mixtures are now under way. The present results indicate that variations in number of adventitious buds formed can be effected by various gas mixtures.

*Extracts of stem tissue.* Several attempts have been made to extract from cuttings natural substances which appear to regulate growth. From *Hibiscus* cuttings we obtained both growth-accelerating and growth-inhibiting substances. Alcoholic extract of the wood alone caused epinasty of

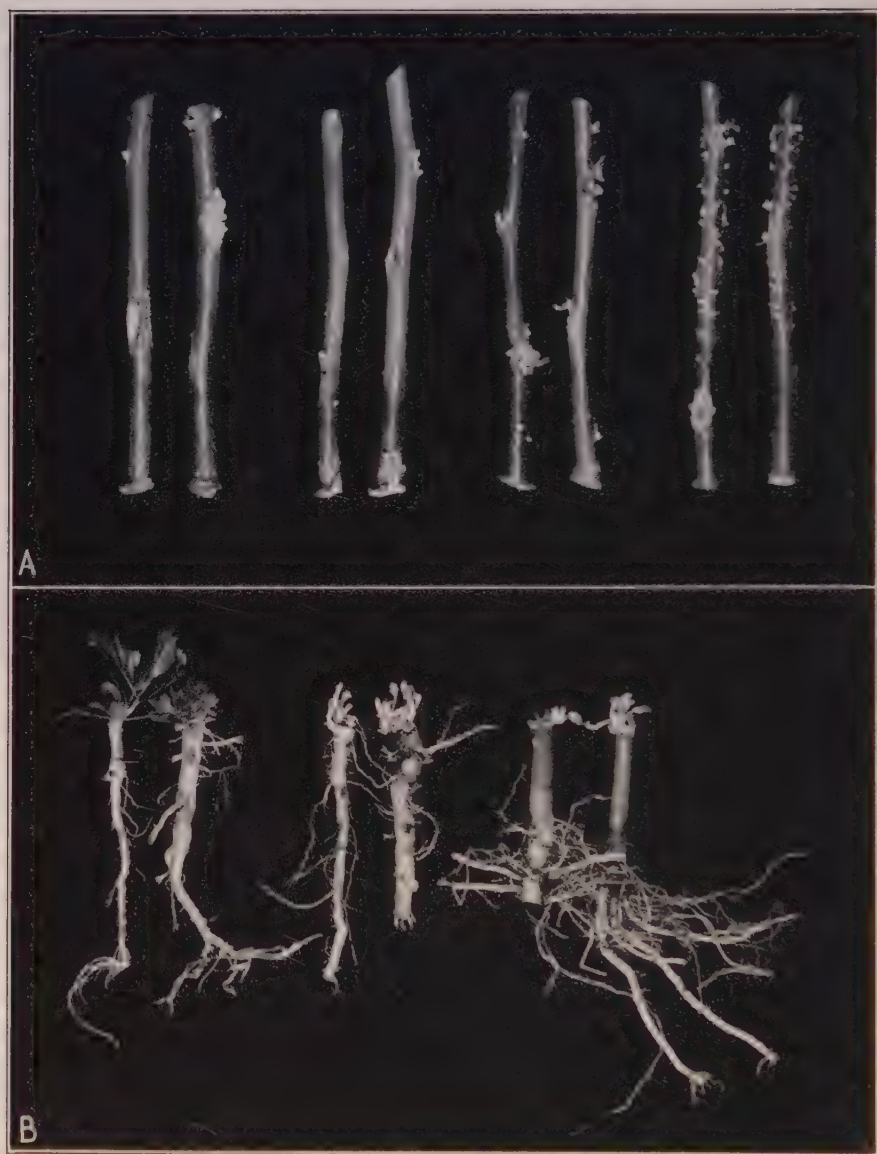


FIGURE 5. *Althea* cuttings showing adventitious shoots. A. Disbudded stem cuttings exposed for five days to abnormal air mixtures and then all placed in moist air. Left to right: air control, high oxygen, high nitrogen, high carbon dioxide. The high concentration was about 90 per cent. Note the carbon dioxide lot formed most adventitious buds. (Photographed after 15 days.) B. Root cuttings producing adventitious shoots at apical end and roots at basal end. Lot at right has stem tissue at top. Note strong polarity shown by root cuttings.

leaves of tomato and later induced roots similar to those induced by the synthetic growth substances. The ether and chloroform extracts of bark varied considerably but often caused positive curvatures when applied to stems and leaves of tomato, indicating inhibition of growth. Other methods for extracting the tissue are now being used and the results will be reported later.

#### DISCUSSION

The capacity of some species of plants to regenerate lost organs from isolated parts has been known for a long time. The subject of regeneration has been frequently involved in botanical and horticultural literature. The organs most often considered are roots but adventitious shoots are also frequently mentioned. The authors of this short paper would not be justified in attempting to review all the literature on the subject. We have elected, therefore, to mention only a few of the papers pertaining to adventitious buds. A recent review of the subject by Prevot (14), citing 116 references will be found valuable by students in this field.

Jørgensen (9), interested in obtaining plants with polyploid number of chromosomes, used decapitated *Solanum nigrum* plants which regenerated shoots after axillary buds had been removed. He states that on the cut surface a thin layer of cork is first formed and then callus tissue from which shoots are initiated, forming a whole bush on the top of the plant. As one batch was removed another started. The procedure could be repeated ten times before the stock got too old. Povolochko (13), working with *Nicotiana* also to obtain polyploid plants, reported that the number of adventitious shoots produced by decapitated plants could be increased by applying orchid pollen to the cut surface. He concluded that when the growing tip was removed the natural supply of hormone was eliminated and that the pollen substituted for the natural substance. Hachaturov (7) found that best regeneration of disbudded *Nicotiana* occurred between the first and fifth leaf. Greenleaf (6) applied indoleacetic acid to the cut surface of disbudded plants and reported favorable effects for bud regeneration of *Nicotiana* but not for tomato.

Simon (15) performed a large number of experiments with cuttings of *Populus nigra* and showed that the apical callus normally formed adventitious shoots and basal callus formed roots but under certain conditions this could be reversed to some extent. Fischnich (5), working with *Populus*, claimed that a 0.25 per cent solution of sodium indoleacetate applied to the callus induced roots and the same substance would induce shoots if present in low concentration.

Chouard (3) working with leaf cuttings of species propagated by leaves, controlled the place where roots and shoots originated by proper incisions, orientation to gravity, transpiration modifications, and the use of indole-



acetic acid. He concluded that "auxins" are not "rhizocalines" or agents of organogenesis but that they cause proliferations and then other factors are responsible for the organization.

Bouillenne (2) and others (14) in his laboratory have long been associated with studies involving organogenesis. In recent studies with *Begonia rex* leaves, they showed that indoleacetic acid does not inhibit formation of adventitious buds and that anaerobic conditions favor their formation.

Stoughton and Plant (16), working with sea kale (*Cambe maritima*), stated that the results of their experiments suggest that, "whatever other substances may be concerned in bud and root production, as proposed by Went, not only the subsequent growth, but also the initial differentiation of meristematic tissue is determined at least in part by the local concentration of the growth substance."

Beal (1), working with decapitated plants of *Lilium harrisii* treated with indoleacetic acid, showed that the region of visible response to the treatment is limited largely to the cells of the epidermis and outer cortex in the immediate cells of the leaf axil. Neither buds nor bud primordia were present in the upper leaf axil at the time of treatment. The meristematic cells in the leaf axils undergo repeated cell divisions resulting in a hump of cells which becomes a bud. From one to three buds may develop in one axil.

Crooks (4) and Link and Eggers (11) have shown that adventitious shoots arise from epidermal cells of flax hypocotyls when the seedlings are decapitated just below the cotyledon leaves.

Lindner (12), working with transverse segments of horse-radish roots, showed that buds and roots were produced from the sides in association with small lateral root traces. Buds formed at the morphological top and roots at the morphological bottom regardless of the orientation of the segment. Naphthaleneacetic acid inhibited the bud formation, but stimulated roots.

Howard (8) reported that an axillary bud of *Brassica* was converted into a root by treatment with indoleacetic acid. He suggests that at first there is development of a meristem and then a root-determining influence on this meristem.

White (17) called attention to the production of buds by callus of *Nicotiana* being cultured after the clump sank into the nutrient solution. He suggested poor aeration as a possible influence.

Levine (10) reported the growth of roots and shoots from galls induced on several species of plants by synthetic growth substances and carcinogenic agents. He has reviewed the subject thoroughly, using many references, and no further citations in this field will be given here.

Many references could be given to support the claims that growth substances have the capacity to induce roots. Adventitious roots can be induced to form on leaves, stems, roots, and even floral parts by local appli-



cation of these formative substances. Systemic responses involving roots on all organs at one time can be induced if the entire plant is exposed to vapors of the active substances. With these facts at hand the logical reasoning might well be that other organ-inducing substances must exist.

From the references cited above it can be seen that a few claims have been made for indoleacetic acid as a bud-forming substance. None of the results, however, shows clearly that buds were induced in the same sense that roots can be induced. The most that can be safely said is that under certain conditions tissues which could be induced to form shoots may under proper influence form roots instead and vice versa. That, however, should not be interpreted to mean that one and the same substance can do both. It is not likely, as the present authors have repeatedly stated, that one substance acts alone to bring about hormone-like responses. It is more logical to assume that a chain of substances must be involved. When any link is missing, the response cannot occur. Substitutions can be made with other chemicals.

Generally speaking, most authors agree that root-inducing substances inhibit initiation of adventitious shoots. The results reported in this paper show that effect clearly for the indole and naphthalene substances but not for the phenyl compounds. These latter substances might be found to play a very important rôle which is not yet understood. They vary in many ways from the most active root-inducing substances (18).

From the bark of althea we have extracted both growth-accelerating and growth-inhibiting substances. Neither has been well purified and perhaps cannot be until better methods have been developed. The fact that both substances are present suggests an interesting physiological balance under normal conditions. The possibility of growing shoots on wood separated from the bark seems to offer new lines of attack in attempts to locate active shoot-forming agents. The isolated wood did not produce roots. However, extracts of the wood induced leaf epinasty and roots when applied to tomato stems.

It has been reported that adventitious shoots frequently possess a polyploid number of chromosomes (9, 13, 7). From 1 to 10 per cent or more of the adventitious shoots of *Nicotiana* and tomato are said to be polyploid. The question arises as to whether the variation might be due to the variations in tissues giving rise to the shoots. Our work indicates that shoots might arise from bark or callus of intact cuttings and from either bark or wood when these are separated. If certain tissues give rise to the polyploid condition the new method of growing adventitious shoots on isolated portions of the stem might offer a good means of studying the polyploid problems.

There is an interesting suggestion in the fact that varying the proportion of carbon dioxide in the air caused an increased number of adventi-

tious buds to form on althea cuttings. This recalls the suggestion by White (17) of poor aeration as a factor and of Prevot (14) that anaerobic conditions favor the formation of adventitious organs in *Begonia rex*.

Both root and stem tissue of althea have the capacity to regenerate shoots and roots. With root pieces stronger polarity is evident than with stems (Figs. 4 A and 5 B). The presence of roots on a stem does not prevent adventitious shoots from forming at the apical end. The origin of the determining influence is not understood.

#### SUMMARY

This paper considers the capacity of several species of plants to produce adventitious shoots and roots under natural influences and the effects of applying growth substances, mutilating the tissue, and varying the external conditions.

Althea cuttings produced numerous adventitious shoots along the dis-budded stem in contrast to cuttings with normal buds intact. Most of the new buds were initiated from the upper end of the cuttings although in two-year-old tissue they extended to within an inch of the base. The lower end of the cuttings produced adventitious roots. There was a relatively narrow transition zone between the regions producing shoots and those producing roots. The new organs were usually associated with proliferating lenticular tissue or on the callus formed at scars. The results suggested that potential meristematic tissue was first stimulated into activity and then regulated by a chemical influence.

The well known root-inducing substances prevented the initiation of shoots and instead induced roots from the same tissues. When disbudded cuttings of althea were treated with vapors of naphthalene and indole substances, roots were induced from upper, middle, and lower regions, thus disturbing the normal polarity in contrast with controls which had shoots on the upper part and roots at the base.

By separating bark from the wood and otherwise mutilating cuttings, it was found that wood kept under humid conditions produced callus and then adventitious shoots from this new tissue but not roots.

Extracts of the bark contained both growth-accelerating and growth-inhibiting substances. Extracts of the wood induced leaf epinasty and roots when applied to tomato plant tissue.

In contrast to althea, disbudded cuttings of *Populus* and *Elaeagnus* produced adventitious shoots only at the apical cut surface or at the scars where buds were removed.

Cuttings of althea, exposed for a few days to high concentrations of carbon dioxide or traces of ethylene gas and then stored in the moist chamber, produced more adventitious buds than controls. It is suggested that these gases interfere with the normal metabolism and bring about a condition that favors the initiation of adventitious organs.

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# EFFECTS OBTAINED WITH MIXTURES OF ROOT-INDUCING AND OTHER SUBSTANCES

A. E. HITCHCOCK AND P. W. ZIMMERMAN

## INTRODUCTION

In a previous brief report (5) data were given showing that mixtures of certain root-inducing substances were more effective for root formation in cuttings than equivalent concentrations of the individual substances. It is the purpose of the present paper to give a more detailed account of these results and to report additional data relating to the use of root-inducing and accessory substances, particularly vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub>, and nicotinic acid.

## MATERIALS AND METHODS

*Talc preparations.* Stock talc and talc preparations containing root-inducing substances or vitamins, or both, were obtained in a mixed and finely-ground state from Merck & Co. Inc., Rahway, New Jersey. Mixtures made from stock preparations were prepared by placing measured quantities of the ingredients in bottles which were rotated for 30 minutes on a machine. It was found previously that these specially prepared talc preparations were considerably more effective than those prepared by mixing ordinary commercial grades of root-inducing acids and talc (4).

*Solutions.* Dilute solutions of less than 100 mg./l. were so prepared as not to contain more than 0.01 per cent ethyl alcohol in the tap water carrier. Concentrated solutions ranging from 2000 to 8000 mg./l. contained 50 per cent ethyl alcohol by volume. Control cuttings were treated with 50 per cent alcohol.

*Successive treatments.* In the case of talc preparations the basal ends of cuttings were treated as follows: dipped to a depth of about one-half inch in the first powder, the excess powder tapped off, dipped into the second preparation, the excess powder tapped off, and then the cuttings were planted.

*Ethylene treatments.* Tap water was saturated with ethylene at a temperature of 5° C. The flask containing the solution was immersed in ice-water until all aliquots had been removed. Test solutions containing ethylene were held at room temperature (24° C.) during the period of treatment.

*Vitamins.* The crystalline vitamins used in the solution tests were obtained in the form of the hydrochloride.

*Test plants.* Cuttings of the following plants were used: *Actinidia arguta* Miq., *Celastrus orbiculatus* Thunb., *C. tartarinowii*, *Chrysanthemum* sp. var. Red Bird, *Euonymus radicans* Sieb., *Hibiscus syriacus* L., *Ilex crenata*

Thunb., *I. opaca* Ait., *Ligustrum ovalifolium* Hassk., *Lycopersicon esculentum* Mill., *Paeonia suffruticosa* Andr., *Picea abies* (L.) Karst (*P. excelsa*) var. Barryi, *Sciadopitys verticillata* Sieb. & Zucc., *Sequoia gigantea* DC., *Taxus cuspidata* Sieb. & Zucc., and *Thuja occidentalis* L. var. *globosa*.

*B<sub>1</sub>*—soil tests. The following potted plants were watered weekly with concentrations of 1 and 100 p.p.m. of *B<sub>1</sub>*: *Begonia semperflorens* Link & Otto, *Camellia japonica* L. vars. *alba plena*, *chandleri elegans*, and Sarah Frost, *Capsicum frutescens* L. (pepper), *Coleus blumei* Benth., *Crassula rubicunda* E. Mey., *Dahlia variabilis* Desf., *Euphorbia fulgens* Karw., *Fuchsia hybrida* Voss., *Gardenia* sp. var. Hadley, *Hedera helix* L. (ivy), *Hibiscus rosa-sinensis* L., *Ilex aquifolium* L. (holly), *Kalanchoe daigremontiana* Hamet et Perrier, *Mimosa pudica* L., *Narcissus tazetta* L. var. Paper White, *Nicotiana tabacum* L. (tobacco), *Pelargonium* sp. (geranium), *Rosa* sp. vars. Briarcliff and Golden Climber (rose), *Saxifraga sarmentosa* L. (strawberry-geranium). The soil used in these tests was a sod soil of this region which had been composted according to the usual practice and hence was not lacking in the principal mineral fertilizer elements or in organic matter.

The following plants were grown in quartz sand supplied with Shive's modified complete nutrient solution in addition to the weekly treatment with vitamin *B<sub>1</sub>* in concentrations of 50 and 100 p.p.m.: *Camellia*, *Fuchsia*, *Gardenia*, *Ilex*, and *Mimosa*.

All potted plants receiving weekly applications of *B<sub>1</sub>* were grown in a house furnished with four hours of extra light each day from December 9, 1939 until February 19, 1940, at which time measurements of the height and length of new shoots and records of flowering and general appearance were made. All plants exhibited active growth some time during the period mentioned.

*Explanation of symbols.* The principal root-inducing acids are referred to by the following symbols: indoleacetic (IA), indolebutyric (IB), and  $\alpha$ -naphthaleneacetic (NA). Symbols for the derivatives of these acids are KIA and KIB for the potassium salts of indoleacetic and indolebutyric acids respectively, and MIB for the methyl ester of indolebutyric acid. Other symbols are PA for phenylacetic, IP for indolepropionic, and P for *p*-phenylenediamine.

## RESULTS

### ROOT-INDUCING SUBSTANCES

#### *Talc Preparations*

*Mixtures.* In one of the principal experiments three mixtures of two different substances (IB-NA, IB-IA, and IA-NA) were used in five different proportions (0:100, 25:75, 50:50, 75:25, and 100:0 per cent) and three different concentrations (2, 5, and 12 mg./g.). These preparations were applied the same day to cuttings of *Chrysanthemum*, *Euonymus*, *Hibiscus*,

and privet (*Ligustrum ovalifolium*). Summary data for this experiment appear in Figures 1 and 2. Illustrations of some of the rooted cuttings are shown in Figures 3 A and 3 B.

Curves for the root counts in Figure 1 are based on the average values for all three concentrations (2, 5, and 12 mg./g.). Thus these curves show the influence of varying the percentage of the two substances at a given concentration. The percentage of the first named substance in all mixtures increases from left to right in steps of 25 per cent and the second named

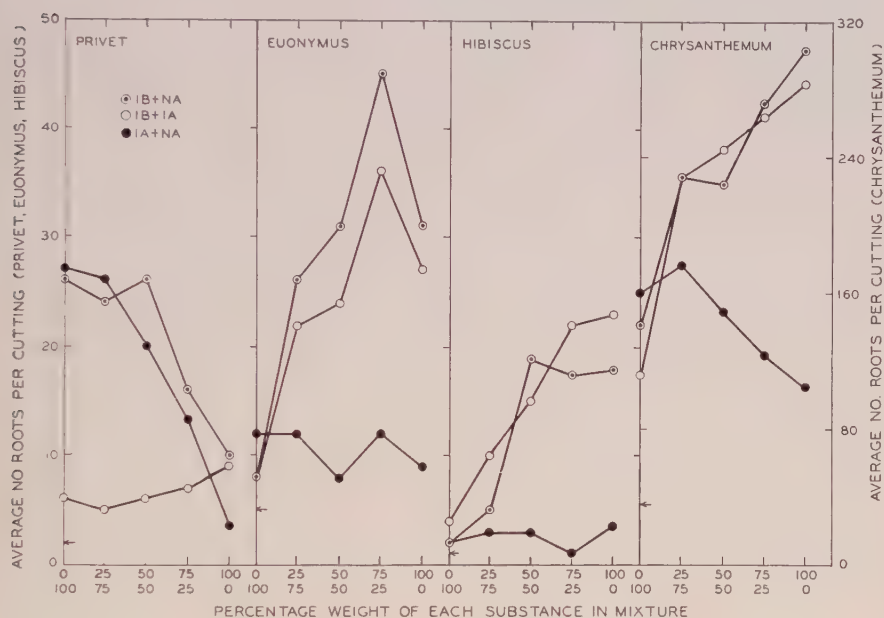


FIGURE 1. Effect on root formation of varying the proportions of two root-inducing substances in a talc preparation. Each point represents the average value for three concentrations (2, 5, and 12 mg./g.).

substance decreases proportionally so that the sums of the two are in all cases equal to 100 per cent. The first and last datum points represent values for 100 per cent of the individual substances. Thus the relative positions of these two terminal points along the ordinate axis is a measure of the relative difference in activity for the two substances used in each mixture. For example, the curve for the IA-NA mixture (privet) shows that IA alone (100 per cent IA) induced an average of 4 roots per cutting and NA alone (100 per cent NA) induced an average of 27 roots. The mixtures of IA and NA (25:75, 50:50, and 75:25 per cent of IA and NA respectively) gave values intermediate between 4 and 27, and approximately propor-

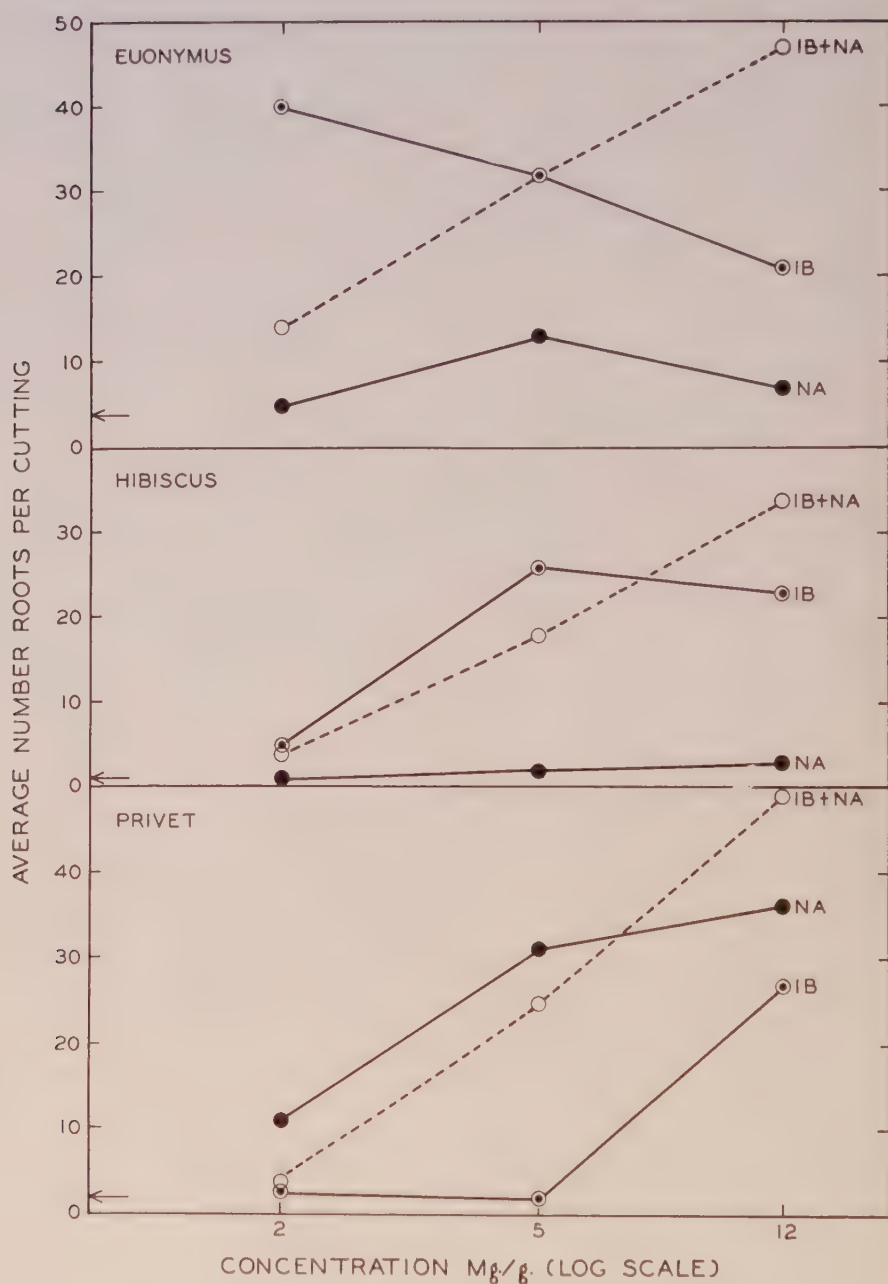


FIGURE 2. Effect of concentration on root formation in cuttings treated with individual substances and with mixtures of equal parts of the two substances.



tional to the relative activities and amounts of the two substances in the mixture. Similar results were obtained with the IB-NA mixture and hence the root count curves for the two mixtures containing NA are approximately parallel and of nearly the same slope.

In contrast to the proportional influence of two substances of unequal activity (IA and NA or IB and NA) for root formation in privet cuttings, the root count curve for the IB-IA mixture shows the influence of two substances of about equal activity for privet. Although IB was slightly more effective than IA, the quantitative difference is small and hence mixtures of these two substances in any proportion are of about equal activity on privet cuttings.

Root count curves for *Euonymus*, *Hibiscus*, and chrysanthemum show essentially the same trends as those described for privet, with the important exception that IB was considerably more effective than NA or IA. In the case of chrysanthemum IB was much more effective than IA or NA, and NA was considerably more effective than IA. Consequently, in all three mixtures the activity varied according to the proportions of the two substances, so that increasing the proportion of the most active substance caused an increase in the effectiveness of the mixture. The much lower activity of IA as compared with NA or IB was reported previously for chrysanthemum (3, p. 475). It is to be observed that 59 of the 60 values for preparations containing NA, IB, or IA are higher than those for the corresponding talc controls which are indicated by small arrows (Fig. 1).

Although three of the four genera (Fig. 1) were IB-sensitive and one was NA-sensitive, the use of a mixture of equal parts of IB and NA proved highly effective for both types. The higher effectiveness of the mixtures for both IB- and NA-sensitive cuttings as compared with the activities of the individual substances is more clearly shown in Figure 2. Curves for the IB-NA mixture show a more nearly proportional increase in number of roots with increasing concentration as compared with curves for IB or NA alone.

Mixtures of equal parts of IA, IB, and NA, and of 50 per cent of one and 25 per cent each of the other two substances were applied to *Euonymus*, *Hibiscus*, privet, and *Taxus* cuttings at a total concentration of 5 mg./g. In all cases the two types of three-substance mixtures were more effective than any one of the individual substances used at the same concentration (5 mg./g.). Since NA was relatively more effective than IB or IA for root formation in *Taxus* cuttings, increasing the proportion of NA in the mixtures caused an increase in the number of roots induced.

One series of tests involved the use of IB and NA individually and in mixtures of equal parts at total concentrations of 0.5, 1, 2, 4, 8, and 16 mg./g. This is in contrast to the 2, 5, and 12 mg./g. preparations used in the tests described in the preceding paragraphs. The use of the geometric

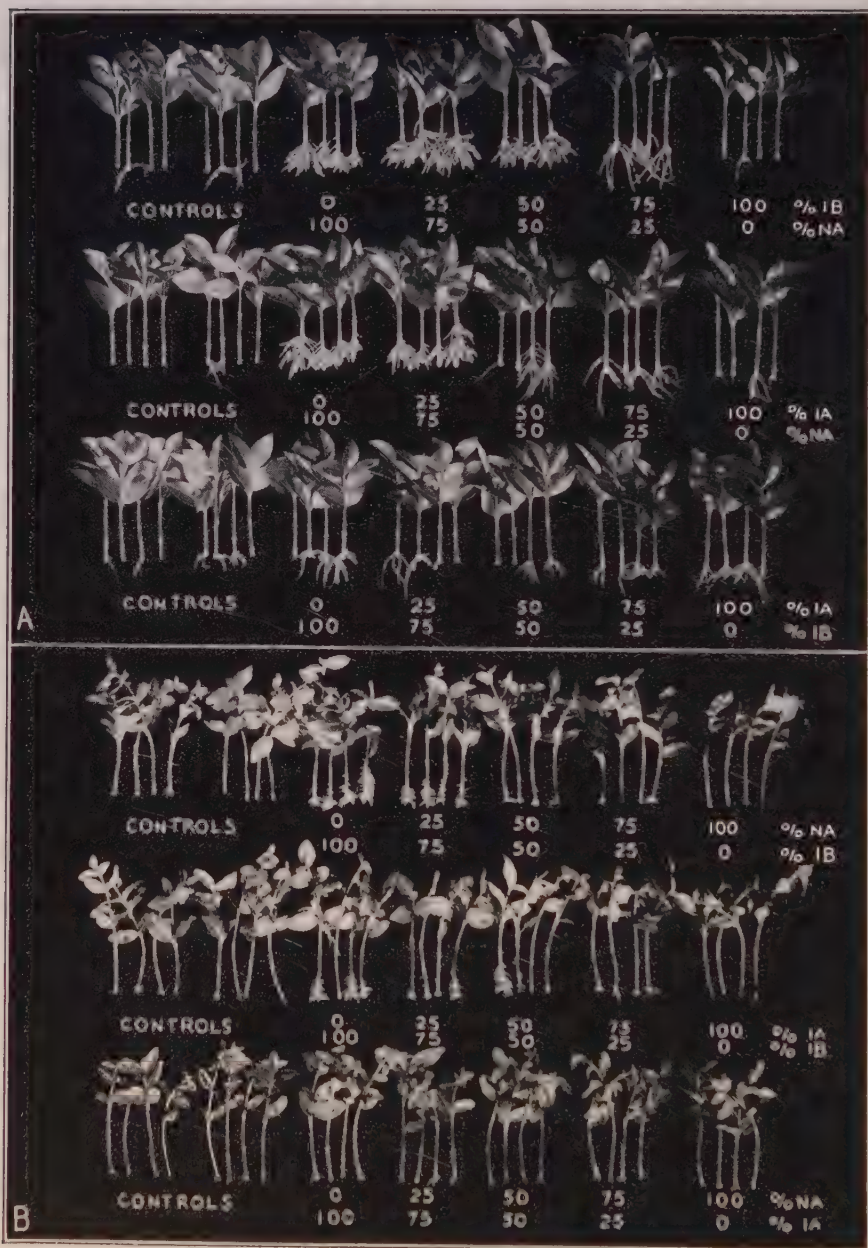


FIGURE 3. Predominating influence of one substance in the presence of another when the mixtures are applied to (A) privet in a concentration of 5 mg./g. and to (B) *Euonymus* in a concentration of 2 mg./g. Two lots on left are non-treated and talc controls respectively.

series mentioned above makes possible a more accurate comparison of responses on the basis of the total concentration of root-inducing substances in mixtures and their individual concentrations in mixtures and when used alone. In addition, the increase in concentration in the geometric series involved smaller steps than in the series 2, 5, and 12 mg./g.

Mixtures generally were effective at lower concentrations and induced more uniform rooting over the effective range as compared with either substance used alone. For example, mixtures of equal parts of IB and NA having a total concentration of 4 mg./g. induced more uniform rooting, a higher percentage of rooted cuttings, and a greater number of roots per cutting in *Celastrus*, *Thuja*, and *Sequoia* than the same concentration of IB or NA used alone.

All species of the same genus did not always respond alike. Whereas the maximum effective concentration of the mixture was 1 mg./g. for *Celastrus tartarinowii*, the corresponding maximum for *C. orbiculatus* was 8 mg./g. For both species IB or NA was much less effective than the mixture with respect to root number, percentage of rooted cuttings, and uniformity of rooting. However, the mixture was effective over a much broader range (2 to 8 mg./g.) for *C. orbiculatus*, indicating that this species is relatively tolerant. Mixtures were relatively more effective than individual substances on cuttings of *Ilex opaca* but not on cuttings of *Ilex crenata*.

NA was more active than IB on *Thuja occidentalis* var. *globosa*, but IB was effective over a broader range, beginning at 2 mg./g. and not reaching a maximum effect even at 8 mg./g. In contrast NA was effective beginning at 4 mg./g. and caused maximum effects at 8 mg./g. The mixture induced maximum rooting at a concentration of 4 mg./g., but at higher concentrations (8 and 16 mg./g.) the reduction in root number was not associated with any noticeable visible injury.

Results with *Thuja* are in marked contrast to the highly toxic effect of the mixtures on most species and varieties of spruce (*Picea*) cuttings in which case part or all of the stem was killed by concentrations of 2 mg./g. or higher. Thus spruce, particularly varieties of *Picea abies*, would appear to be one of the most sensitive of the evergreens. Optimum rooting in cuttings of *P. abies* var. *Barryi* was induced by a 1 mg./g. preparation of IB.

Although emphasis has been placed upon certain favorable effects obtained with mixtures of IB, NA, and IA incorporated in talc, a more than additive effect was not always found. Furthermore, cuttings of the same species or varieties did not respond alike when treated at different times of the year. The failure of *Picea* cuttings to respond has been mentioned. Tree peony (*Paeonia suffruticosa*) rooted about the same regardless of whether the cuttings were treated with root-inducing substances (IB, IA, or NA) individually or with mixtures, or were not treated (controls). In the case of *Sciadopitys*, NA was observed to have an unusual effect, causing pro-



nounced swelling of stems nearly to the tip. The lower part of the NA-treated cuttings increased in diameter from two to three times that of controls. Mixtures containing NA caused the same type of response. Concentrations of the 50 per cent mixture in excess of 4 mg./g. appeared to be injurious to certain types of *Sciadopitys* cuttings. However, only treated cuttings had rooted at the end of four months in the case of cuttings taken in October.

*Successive treatment.* When *Hibiscus* cuttings were treated successively with two substances (NA, IB, or talc) there was a definite predominating influence of the preparation first applied. Each of the two root-inducing substances was used in concentrations of 0, 2, 5, and 12 mg./g. Since IB was applied first in one series of tests and second in another series, there were in all 32 tests. Where IB was applied first and NA second the root counts were 10, 26, 61, and 78 (total of 175) for concentrations of 0, 2, 5, and 12 mg./g. respectively. The corresponding values for IB as a second application were 2, 12, 42, and 58 (total of 114) respectively. As mentioned previously, *Hibiscus* is an IB-sensitive type. Similar results were obtained with tomato leaf cuttings at lower concentrations (0, 0.5, 1, and 2 mg./g.).

In another experiment *Hibiscus* cuttings were subjected successively to different concentrations of the same substance. The first and second treatments with different concentrations of IB were as follows: 2 and 5, 5 and 2; 2 and 12, 12 and 2; and 5 and 12, 12 and 5 mg./g. Corresponding root counts were: 7 and 22; 15 and 18; and 16 and 24, in which the second value in each pair represents treatment with the highest concentration first. Thus in all cases the most effective treatment consisted of applying the highest concentration of IB first.

Results with successive powder treatments indicate that there is a predominating influence of the preparation first applied regardless of whether the same substance was used in different concentrations or different root-inducing substances were used. Applying talc first reduced the effectiveness of a subsequent treatment with IB or NA. However, the two preparations applied successively did not act the same as mixtures since the order of application influenced root number to a greater extent than increasing the total concentration of the two preparations. That portion of the powder which first comes in contact with the cuttings appears to act instantly with the tissue, causing changes which prevent the same tissue from responding as originally. There is the additional possibility that the preparation applied second does not mix thoroughly with the preparation applied first. Perhaps only a relatively small proportion of the second preparation would come in direct contact with the tissue. Failure to obtain a more than additive effect with successive IB and NA treatments similar to that obtained with mixtures is an indication that the two preparations applied successively did not become thoroughly mixed on the cutting. The present re-



sults, as well as those previously reported (4), are in accordance with the idea that the principal action of applied root-inducing substances occurs within a relatively short time—that is, from a few seconds up to about 24 hours, depending upon the method of application.

### *Solution Mixtures*

*Dilute preparations.* Results obtained with mixtures applied as solutions to privet, *Hibiscus*, *Euonymus*, and tomato (leaf) cuttings were similar to those described for talc preparations. Data for tomato leaf cuttings appear in Table I. As indicated by the group total values, the mixtures (part B) were more effective on a total concentration basis than KIB used alone at the same concentration (part A). In the three-substance mixture KIB constituted one-third and in the four-substance mixture one-fourth of the total concentration. Thus the concentration of KIB in the mixtures was 0.33, 1.07, and 3.3 in one case, and 0.25, 0.8, and 2.5 mg./l. in the other case. It is to be noted that these concentrations of KIB were more effective in the presence of other substances than KIB alone in concentrations of 1, 3.2, and 10 mg./l. These results do not appear to be explainable on the basis of the individual activities shown in part A.

That the mixtures were acting in more than an additive manner is further substantiated by the rooting responses recorded in part C of Table I.

TABLE I

ROOT FORMATION IN TOMATO LEAF CUTTINGS TREATED WITH KIB IN THE PRESENCE OF OTHER SUBSTANCES

Substances* in mixture	Total concentration, mg./l.				Group totals
	0	1	3.2	10	
A. Number roots induced by individual substances					
KIB	0	56	57	48	161
KIA	0	0	0	26	26
IP	0	1	8	1	10
P	0	1	1	0	2
B. Number roots induced by mixtures of substances in equal amounts					
KIB	0	56	57	48	161
KIB, KIA, P	0	56	72	61	189
KIB, KIA, P, IP	0	44	80	59	203
C. Distance in mm. roots emerged above base of cutting					
KIB	0	43	50	60	153
KIB, KIA, P	0	45	57	63	169
KIB, KIA, P, IP	0	38	53	68	159

\* KIB = K-salt of indolebutyric acid, KIA = K-salt of indoleacetic acid, IP = indolepropionic acid, P = *p*-phenylenediamine.

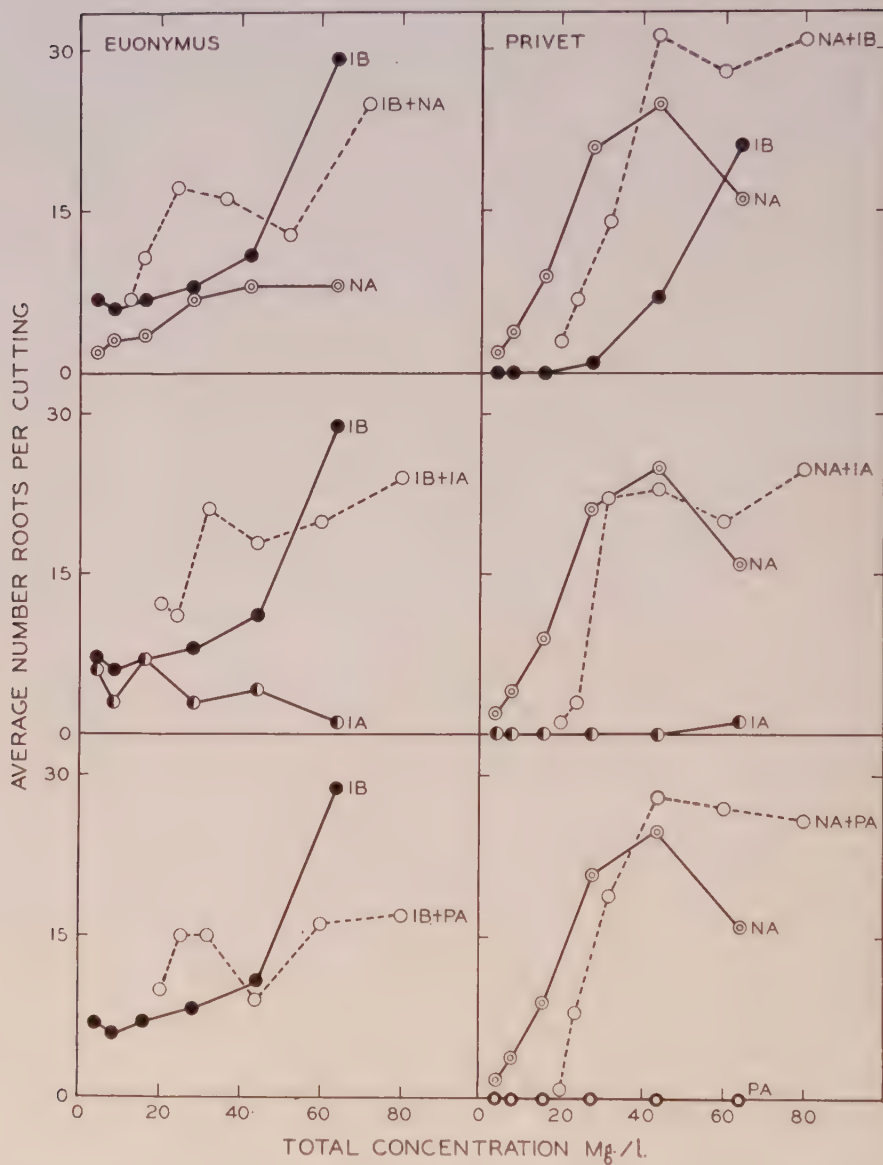


FIGURE 4. Effect on root formation of varying the proportion of one substance (IB for *Euonymus* and NA for *privet*) while maintaining another substance at a constant concentration of 16 mg./l. (*privet*) or 8 mg./l. and 16 mg./l. (*Euonymus*).

Since the distance in millimeters roots emerge above the base of the cutting is a function of concentration (3), the data in part C showing an increase in distance with an increase in concentration indicate that the mixtures were functioning as relatively high concentrations of KIB and not according to the proportion of KIB in the mixture.

In addition to the use of equal parts of two or more substances, another method was to vary the concentration of one substance (4, 8, 16, 28, 44, and 64 mg./l.) while maintaining another substance at a constant concentration of either 8 or 16 mg./l. Mixtures applied to privet contained additions of IB, IA, or PA to a basic series of NA solutions. Corresponding additions for *Euonymus* were NA, IA, or PA, and the basic series of solutions in this case contained IB. Root count values for these tests are shown in Figure 4.

Data for privet (Fig. 4) show that the approximate minimum effective concentration was 8 mg./l. for NA and 44 mg./l. for IB, whereas IA and PA were not effective up to a concentration of 64 mg./l. The three mixtures were more effective and less toxic at concentrations of 44 to 80 mg./l. than NA alone was at 44 to 64 mg./l. Although IA and PA were themselves inactive for root formation in these tests, when they were used in mixtures with NA, the resulting activity was about the same as that for the NA-IB mixture. These results indicate that the action of IB, IA, and PA in the presence of NA was essentially the same for the mixtures applied to privet cuttings.

In the case of *Euonymus* (Fig. 4) the mixtures were less effective in the range 44 to 80 mg./l. than IB alone, but in the range 16 to 44 mg./l. the mixtures were more effective than IB. The relation between the increase in root number and increase in concentration of the mixture was less consistent for *Euonymus* than for privet (Fig. 4). However, it is to be noted that mixtures consisting of equal parts of the two substances and having a total concentration of 32 mg./l. were effective on cuttings of both *Euonymus* and privet. At a total concentration of 32 mg./l. the mixtures applied to privet contained 16 mg./l. of NA and those applied to *Euonymus* contained 16 mg./l. of IB. Since none of the four substances (NA, IB, IA, and PA) was as effective at a concentration of 16 mg./l. as the mixtures were at 32 mg./l., it is obvious that the activity of the mixtures in this case could not be due solely to the NA or IB content. Likewise, the root count values for the mixtures are not additive and hence are not approximately equal to the sums of the values for the two substances used separately; they are more than additive. Similar results were obtained with tomato leaf cuttings. Additional tests with tomato showed that mixtures of IB and MIB were of about the same activity as those for the two substances used separately. In this respect the results are similar to those described for treatment of *Euonymus* and *Hibiscus* cuttings with powder preparations of NA and IA (see Fig. 1).

*Concentrated solutions.* The basal ends of cuttings of privet, *Hibiscus*, *Celastrus*, and *Actinidia* were dipped into solutions containing 2 to 8 mg./cc. of IB, NA, or mixtures of equal parts of the two substances. No consistent more than additive effects were obtained in these tests.

#### ACCESSORY SUBSTANCES

*Talc preparations.* Vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub>, and nicotinic acid were used in concentrations ranging from 0.01 to 10,000 p.p.m. in the presence and in the absence of IB. Results obtained with holly (*Ilex opaca*) indicated that talc mixtures containing 12 mg./g. of IB and one per cent B<sub>1</sub> acted as a more effective preparation than a similar preparation lacking B<sub>1</sub> (Table II). The IB mixture containing B<sub>1</sub> induced a greater number of roots,

TABLE II  
EFFECT OF VITAMIN B<sub>1</sub> ON ROOTING OF DORMANT ILEX OPACA CUTTINGS

Type of Injury administered	Av. No. roots			Root length (mm.)		
	Control	IB, 12 mg./g.	IB, 12 mg./g. B <sub>1</sub> 1%	Control	IB, 12 mg./g.	IB, 12 mg./g. B <sub>1</sub> 1%
None	0	17	32	0	21	8
Lower buds removed	0	30	61	0	44	30
Stem slit near base	0	46	49	0	43	24
Base of stem crushed	0	20	31	0	28	15
Column totals	0	113	173	0	136	77

shorter roots, more swelling and proliferation, and the roots emerged from a greater area of stem tissue as compared with the responses to the IB mixture lacking B<sub>1</sub>. The percentage increase in the average number of roots per cutting in four tests was 7, 55, 88, and 104 per cent depending upon the type of cutting used (Table II). Reduction in root length ranged from 32 to 62 per cent. B<sub>1</sub> alone was inactive for root formation. Thus in these tests B<sub>1</sub> was not acting as a root growth factor.

Talc mixtures containing 2 mg./g. of IB and 0.1 per cent B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub>, or nicotinic acid caused an increase in the number of roots ranging from 26 to 70 per cent for tomato leaf cuttings (Table III). In the case of tobacco leaf cuttings only B<sub>1</sub> and B<sub>6</sub> appeared to be effective (Table III). The increase in root number was 30 and 56 per cent respectively. By themselves, the vitamins were inactive for root formation in tomato and tobacco leaf cuttings. When applied at the start, as in these tests, B<sub>1</sub> failed to act as a root growth factor.

*Solutions.* When tomato leaf cuttings were treated for 24 hours with 10 mg./l. of KIB and then for 24 hours with B<sub>1</sub>, the average number of roots at the end of six days increased with increasing concentrations of B<sub>1</sub> (Table



III). At a concentration of 1 mg./l. of KIB a similar activating effect was not produced by B<sub>1</sub>.

TABLE III  
EFFECT OF VITAMINS ON ROOTING OF TOMATO LEAF CUTTINGS. AVERAGE NUMBER OF ROOTS PER CUTTING

A. Treated with mixtures of KIB* and vitamin in talc					
Substance added (0.1%)	Tomato leaves		Tobacco leaves		Group totals
	KIB, 2 mg./g.	Talc control	KIB, 2 mg./g.	Talc control	
None	54	11	19	1	85
Nicotinic acid	83	1	21	1	106
B <sub>1</sub>	68	3	27	1	99
B <sub>2</sub>	79	3	14	1	97
B <sub>6</sub>	92	0	43	2	137

B. Treated 24 hours with KIB, then 24 hours with B <sub>1</sub>					
KIB, mg./l.	Concentration B <sub>1</sub> , mg./l.				
	0	0.01	0.1	1	10
1	7	5	10	3	12
10	25	39	50	57	64

C. Successive treatment with KIB and B <sub>1</sub>					
Time B <sub>1</sub> added	Conc. KIB, mg./l.				Group totals
	0	1	3.2	10	
None	0	17	51	64	132
First day	0	50	57	70	177
Second day	0	32	66	70	168
Third day	0	23	56	68	147
Fourth day	0	44	36	39	119
Fifth day	0	29	29	49	107

\* K-salt of indolebutyric acid.

*Successive treatment.* Solutions of KIB and B<sub>1</sub> were applied successively so that tomato leaf cuttings were subjected to each substance for only 24 hours. Root counts in Table III show that B<sub>1</sub> was most effective when applied in the presence of KIB at the start or immediately following (during the second 24 hours) treatment with KIB (group totals). B<sub>1</sub> was not effective on cuttings which had not received an IB treatment, either in these tests or in any of the other tests.

Cuttings of *Taxus cuspidata*, *Celastrus tartarinowii*, *Actinidia arguta*, and *Hibiscus syriacus* were given an initial treatment with root-inducing substances (IB and NA) and a deferred treatment (24 hours) with vitamin B<sub>1</sub> in concentrations of 0.0, 0.01, 0.1, and 1 mg./l. The deferred treatment with B<sub>1</sub> was applied to *Taxus* cuttings after two and four weeks and to the

other genera after one and two weeks. Rooting responses at the end of two and one-half months for *Taxus* and after one month for the other genera showed no essential difference between cuttings treated with  $B_1$  and those not treated with  $B_1$ , either with respect to number of roots or length of roots.

*B<sub>1</sub>—soil tests.* After a period of about two months the potted plants watered weekly with  $B_1$  solutions were in size and appearance no different from the control plants. A number of the plants grown in soil were removed and the roots freed from soil. There was no essential difference in the appearance of the root systems of treated and control plants. The plants in quartz sand are being kept under observation and the details of these results will be reported at a later date.

The brief mention of the potted plant test in connection with the data relating to treatment of cuttings with  $B_1$  is for the purpose of indicating that any general practical use of  $B_1$  for stimulating root growth of established plants appears not to be justified until verified by more substantial experimental data than have been published to date.

*Effect of ethylene.* Saturated water solutions of ethylene with and without the addition of IB were applied to the bases of tomato leaf cuttings. Successive treatments with ethylene-water solutions and IB were also used. Mixtures of ethylene and IB induced rooting at lower concentrations than IB alone. Successive treatment for 20 to 60 minutes with water saturated with ethylene and then with IB for 24 hours induced more roots per cutting than IB alone. It was observed that cuttings treated with ethylene were more turgid than those not treated with ethylene. Some of the treatments with mixtures of ethylene and IB were run in duplicate so as to determine the influence of a combined solution and gaseous ethylene treatment resulting from placing the cuttings under a bell jar during the period of the test. The results indicated that this treatment was at least slightly more effective than when similarly treated cuttings were not placed under a bell jar. Control and treated cuttings placed under bell jars absorbed much less solution than similar cuttings left outside.

#### DISCUSSION

Greater than additive effects were obtained with mixtures composed of one active root-inducing substance and one or more substances of lower or no activity. Thus the substance or substances of lower activity or no activity appeared to function as activators and hence produced a synergistic effect. Such an effect consisted of an increase in number of roots, higher percentage of rooted cuttings, more uniform rooting in different types of cuttings, and increase in swelling and proliferation, roots emerging from a greater area of stem tissue, or one or more of these effects at the same or at lower concentrations and frequently over a broader range of concentration.

Exclusive of the synergistic effects the mixtures of IB and NA were highly effective on both IB- and NA-sensitive plants. This feature alone is of considerable practical importance since it means that a single preparation will be effective on more genera and species of plants as compared with a single concentration of either substance.

With respect to the many possible combinations of different substances, different proportions, and different concentrations, only a few were tried. However, the results indicate that mixtures composed of two or more substances of about equal activity for a given species are not likely to induce greater than additive effects. In general, the two-substance mixtures were as active as the three- or four-substance mixtures. The possible use of a many-substance mixture does not appear feasible at present, but it is believed that there may be found better combinations than the relatively few which have been tried, particularly with respect to the proportions of the substances in the mixture. Although mixtures of equal parts of two substances gave satisfactory results in many cases, it might be advantageous to reduce the proportion of NA below 50 per cent because of its high activity on certain species, including several of the evergreens.

Mixtures of IB and vitamin B<sub>1</sub> produced greater than additive effects similar to those obtained with mixtures of root-inducing substances. These results were not anticipated since, in the literature, B<sub>1</sub> has always been referred to as a growth factor and specifically as a root growth factor for higher plants. Thus through its effect on root growth, B<sub>1</sub> is assumed to exert a promotive influence on vegetative top growth (1, 2, 7). On the other hand, B<sub>1</sub> is reported by Warner and Went as being inactive for root formation (7, p. 7) "since it affects root growth but not root initiation." In contrast, our results indicate that B<sub>1</sub> functioned as a non-specific stimulant or activator for root formation in cuttings when used in the presence of other known compounds such as IB or NA, even to the point of retarding root growth. Other substances such as IA, PA, B<sub>6</sub>, and ethylene functioned as activators similar to B<sub>1</sub>. This interpretation does not discount the importance of the specific effect of B<sub>1</sub> on the growth of either fungi or isolated root tips, but points to the probability that the action and function of this vitamin are more complex than has previously been assumed. In fact, as our knowledge of growth-regulating substances increases, the subject of growth regulation in general becomes more complex.

Tests with potted plants also failed to show any favorable influence on root growth or top growth as a result of watering the soil weekly with concentrations of B<sub>1</sub> ranging from 0.01 to 10 p.p.m. On the basis of these results any general practical use of B<sub>1</sub> for soil treatment would not be justified. The use of B<sub>1</sub> or other vitamins in mixtures of root-inducing substances may possibly have some practical application but the composition of such mixtures needs further study.

Considering our results with cuttings and established plants, it appears that special conditions are required to demonstrate favorable effects of  $B_1$  on root growth such as appear in the literature. One of the principal examples cited in the literature (1, 2, 7) is the *Camellia*, which is reported as failing to grow without the addition of  $B_1$ . Our results and the widespread occurrence of *Camellia* plants in the South indicate that the culture of this plant is not a special problem. With respect to cuttings, a few varieties (e.g. *alba plena* and *chandleri elegans*) are difficult to root, but they respond readily to treatment with an effective root-inducing substance such as indolebutyric acid (4). It is suggested that the failure of others (1, 2, 7) to root *Camellia* cuttings was not due to a lack of  $B_1$ , as was believed to be the case, but rather to the ineffectiveness of the root-inducing substance used (indoleacetic acid).

It was suggested by Warner and Went on theoretical grounds (7, p. 5), that IA, IB, and NA should be of equal effectiveness for root formation in all species of plants, but they furnished no comparative data. In contrast, our results show that the relative activities of IB, IA, and NA vary considerably according to the genera or species of plants (Fig. 1). Thus, when two of these substances were found to be of distinctly different activity for a given species, mixtures of them varied in activity according to the proportions of the two substances in the mixtures. On the other hand, substances of about equal activity could be mixed in any proportion without changing the activity of the mixture. In no case have we found that NA, IB, and IA are of equal activity individually and in mixtures when applied as solutions or as powders to a given species.

In view of the different classes of substances which functioned as activators (IA, IB, NA, PA, ethylene, and vitamins  $B_1$  and  $B_6$ ) in our tests, it is of interest to consider the interpretations given by Michener for similar effects obtained with ethylene and IA (6) and by Went for similar effects obtained with PA and IA (8). The greater than additive effect obtained with ethylene as the activator was used by Michener as a means of showing that ethylene was not a phytohormone because an exactly additive effect was not obtained in combination with IA. Went (8) concluded that, because PA was itself inactive for root formation in his tests but when followed by treatment with IA more roots were induced than with IA alone, such a method could be used for distinguishing between active and non-active substances for root formation. The reverse order of IA and PA treatment or the use of mixtures of these two substances was not considered by Went. Applying Went's interpretation to our results it could be said in one case (Fig. 4) that both IA and PA were not active for root formation, but only for "pretreatment," since either substance in the presence of NA produced more roots than NA alone, but when used individually both IA and



PA were inactive. However, under other conditions IA was shown to possess activity for root formation.

Considering the favorable effects of certain mixtures on root formation in cuttings, it would seem that additional tests should prove of value. With respect to the possible activating or synergistic effects obtained with mixtures, this rather neglected field would appear to offer especial promise in its application to biological problems in general, and to growth-regulating substances in particular.

#### SUMMARY

The root-inducing activities of mixtures of two or more substances were compared with the activities of the individual substances. Successive treatments were also used. Water or talc were the principal carriers.

In many but not all cases, the mixtures were more effective on cuttings than any of the individual substances. This greater than additive effect was characterized by an increase in number of roots, a higher percentage of rooted cuttings, more uniform rooting, and other effects typical of a higher concentration of a root-inducing substance. Such effects were obtained with substances of different root-inducing activity but not with substances of about equal activity. All genera or species of cuttings did not respond equally well to a given mixture. Certain mixtures of IB and NA were effective on IB- and NA-sensitive cuttings.

Depending upon the kind of cutting and the conditions of the test, the following substances function as activators: IA, IB, NA, PA, vitamins B<sub>1</sub> and B<sub>6</sub>, and ethylene. Only a few of the many possible combinations were tried with respect to number and kind of substances, their relative proportions, and the total concentration. A mixture composed of equal parts of two substances (e.g. IB and NA) proved as effective or more effective than three- and four-substance mixtures.

In these tests vitamins B<sub>1</sub> and B<sub>6</sub> functioned as activators for root formation in cuttings and not as root growth factors. Parallel tests with established plants likewise failed to show any favorable effects of B<sub>1</sub> as a root growth factor when added weekly to the soil of potted plants in concentrations of 0.01 to 10 p.p.m. Deferred treatments of B<sub>1</sub> were not effective on cuttings of tomato, *Taxus*, *Celastrus*, *Actinidia*, and *Hibiscus*, regardless of whether or not the cuttings had been given an initial treatment with a root-inducing substance. It is concluded that before any general practical use of B<sub>1</sub> can be recommended for a soil amendment, more substantial data are needed than have been published to date.

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## LAMELLATE STRUCTURE OF CELLULOSE MEMBRANES IN COTTON FIBERS<sup>1</sup>

FLORENCE L. BARROWS

The earliest microscopic observations on the structure of the cotton fiber were made by Leeuwenhoek in the 17th century (17). He observed that the hairs were flat and had sharp edges on the sides. This observation was communicated by letter to the Royal Society of London in 1684 (41). Early drawings made from microscopic observations by Koechlin for Heilmann (36) and by Bauer for Thomson (58) were published in 1828 and 1834, respectively. As early as 1852 Reissek (50) made both a microscopi-

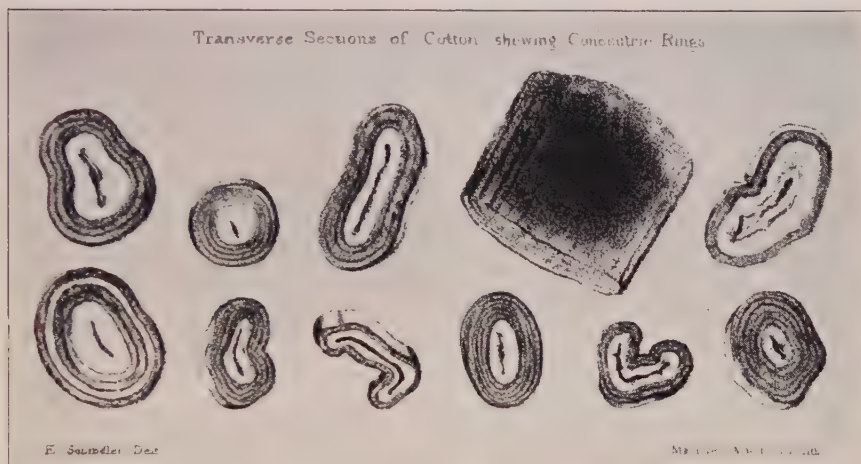


FIGURE 1. After Crum, Plate VII, 1863.

cal and chemical study using the iodine and sulphuric acid test for the presence of cellulose in the cell wall of cotton fibers of two species of *Gossypium*. He was one of the first to report examination of cotton fibers on the day of flowering and also in the unopened boll. In Reissek's (50, p. 178, Taf. LVIII, Fig. XIV) longitudinal view of fibers of *Gossypium barbadense*, wall or membrane layers are clearly shown and at least nine may be counted. Reissek used the term *lamella* in describing these layers.

Crum (25), whose work covered the years 1843-1863, seems to have been one of the first to study cross sections of cotton fibers. His Plate VII (25, p. 414) shows transverse sections of mature cotton with four or five concentric layers, with a legend indicating that the layers are an effect of diffraction, not of structure. This illustration is reproduced in Figure 1.

<sup>1</sup> Cellulose Department, Chemical Foundation, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York.

Although Crum did not quite trust his own observations, it is interesting to note that Bowman (18, p. 436) afterwards studied Crum's original slides, and reported 45 years later, in 1908, a distinct appearance of lamination.

Others who noted the lamellate structure of cotton fibers in longitudinal or cross sections were Butterworth (20), Minajeff (42), Herzog (37), Balls (11), Willows and Alexander (61), Denham (28), and others. Herzog (37) after treating Egyptian Mitafi combed fibers with cuprammonium hydroxide solution for 14 hours reported the presence of as many as 28 lamellae. The number of lamellae and their width have been points over which there has been much controversy.

Balls (11, p. 543) after examining 12 fruits which had been preserved for 5 years and 4 months in "acetic-absolute" published his paper on *daily "growth-rings,"* likening them to annual rings in a tree trunk. He reported 3 rings for 30 days, 5.3 for 33 days, 6.6 for 36 days, 13 as the mean for 39 days, and from 7 to 14 in 42-day fibers; and 50-day mature cotton as having 20 to 25 rings. Fuzz hairs had 16 rings according to him. He used drastic methods of swelling, notably cuprammonium hydroxide, and came to the conclusion that there was a daily formation of growth rings. A footnote in regard to Minajeff's (42, Figs. 4 and 5) illustrations, says Minajeff probably noticed only 4 or 5 out of 2 dozen. In Balls' Figure 8 (11, p. 546) one can count 10 lamellae at the tip. Figure 10 of the same fiber at the base shows 15. His plate on page 555 shows 14 to 16. These are all longitudinal views and not cross sections. The size of the fibers was apparently not measured before or after swelling, but was swollen about 5000 to 9000 diameters, from which it was deduced that the concentric layers were (11, p. 542) laid down during the active growth of each successive night and numbered about 25 in all. Each layer was described as approximately 0.0004 mm. in thickness and it was further stated that the layers were probably ultra-microscopic. These conclusions seem to be unwarranted by the data presented, but have been widely quoted and accepted.

Willows and Alexander (61) worked on a method of preparing cross sections, and their figures show clearly at least six layers. They had on many occasions seen in their sections what Balls considered to be daily growth rings. The age of their fibers was unknown, so they were unable to correlate age with number of rings. They suggested that the concentric layers in Crum's figure (25, Plate VII) might well be the daily growth rings and pointed out that if they were the result of diffraction there should be fringes outside the figures, which were absent.

Denham (27) proposed a new method for cutting cross sections of cotton hairs and called attention to Balls' use of cuprammonium hydroxide which he had found had a tendency to contort the growth ring strata. Balls himself made practically the same statement two years earlier (11, p. 544). Balls and Hancock (13) a few days later reported having used the Denham



method for some months, having found it most satisfactory. They were critical of the method of Willows and Alexander (61). Balls (12) soon after said that Willows and Alexander probably had a fuzz hair and a diffraction effect. Willows, a physicist (60), answered that before publication they had made very sure that they were not dealing with a diffraction effect. They had found that the rings dyed deeply, and that swelling caused the layers to separate. He pointed out that diffraction rings neither dye nor separate and whether the rings are, in fact, growth rings should be decided not by opinion but by the evidence produced.

Although Denham (27) had pointed out the dangers of using cuprammonium hydroxide, Balls and his colleagues continued to use it, and obtained evidence of structure described as "erratic anastomosing series," as have Bailey and Kerr (10, p. 280), Anderson and Moore (6, p. 507), Bailey (9, p. 43), and Anderson and Kerr (3, p. 50).

Farr (31, 32), Compton (24), and Sisson (56) have shown that cuprammonium hydroxide has a differential effect upon the two main components of cellulose membranes. The colloidal cementing material around the cellulose particles and between the lamellae swells, but there is no change in the size of the cellulose particles.

Denham (28, p. T104) saw as many as 20 rings and (p. T106) noted that the fiber base was thicker than the tip.

El Kelaney and Searle's paper (29) has a figure with about 15 layers in cross section. Haller (33, Fig. 5) shows about 12 lamellae clearly visible in a longitudinal view of a cotton fiber. Osborne (47) reported 13 to 17 lamellae in fuzz hairs and 3 to 7 in lint, in treated longitudinal section. Baranov and Mal'tsev (15, Plate 45) showed 4 to 6 lamellae in cross sections.

The workers of the Shirley Institute in England published a number of papers on cotton which dealt with various problems related to the textile industry, among which were changes in sections of cotton hairs on mercerization (21); the rigidity of cotton hairs (48); area of cross sections (23); morphology of the wall (28); and physical cause of lustre in cotton (1), which is due to the shape of the cross section, the thicker-walled circular sections being more lustrous. Data were accumulated as to the length and diameter of cotton fibers and measurements of wall thickness. These various studies included several species of *Gossypium* and a great number of varieties from the main cotton growing regions of the world.

#### PROBLEM

The present report covers a study of the development of lamellate structure in cellulose membranes from day of flowering to maturity in the epidermal hairs of the seed coat of *Gossypium hirsutum* L., a genetically pure line of variety Super Seven. The material was grown in the greenhouse and in the constant light room at the Boyce Thompson Institute

(8). Mature field-grown samples were used for comparison. Attention was focused on cell wall structure, and especially on the number and thickness or width of the lamellae. The earlier stages were studied in the fresh and living condition as well as in paraffin sections.

#### METHODS

Several methods have been employed by different authors for preparation of cross sections of cotton fibers. For younger fibers, up to about the 20th or 25th day after flowering, the paraffin method is satisfactory. For older or mature fibers with thickened walls, modification of the gelatin or a similar method is usually employed, such as Denham's celloidin-paraffin method (27), or the cellulose-acetate method of Willows and Alexander (61). Kisser and Anderson (40) published still another variation of the celloidin-paraffin method. El Kelaney and Searle's acid method (29) seems to have had few advocates.

The various embedding methods used by other workers did not seem to meet the requirements for this problem. Gum arabic (34, 53), celloidin, collodion (38), and parlodion were rejected when they were found to contain cellulose particles. Pure gelatin was selected for embedding material because of its freedom from contamination with cellulose. Gelatin also has the advantage of being non-doubly refractive in polarized light as contrasted with cellulose.

The seeds were usually selected from the middle or base of the unopened boll. After leaving a piece of the seed coat attached and orienting the fibers as nearly parallel as possible, they were tied onto a cardboard reel, so that one could later identify the base and tip of the fibers. The moist and fresh fibers were tied with a colored thread over a window in the cardboard reel, and the reels placed successively in three concentrations of melted gelatin in a 52° C. paraffin oven. After an hour or two in 4 per cent, and about an equal period in 10 per cent, the reels were usually allowed to stand in 25 per cent gelatin overnight. The next morning the reels were embedded in paper trays in 25 per cent gelatin to which a few drops of glycerin were added. When hard and firm the gelatin blocks were immersed in a mixture of one part formalin (40 per cent formaldehyde) to seven parts of water. This hardened the gelatin still more, but kept it moist. The gelatin blocks were stored in this formalin-water mixture in corked glass vials until needed for use. The blocks keep indefinitely when stored in this way. The bundles of fibers were cut away from the cardboard blocks just before hand sections were made for microscopic examination. The sections were mounted in water for study. By addition of a drop of glycerin these could be kept moist indefinitely. By this method the fibers were not allowed to dry out from the moment they were taken moist from the boll. In dry or mature fibers it is more difficult to see the lamellae or layers in cross sec-

tion, but in the gelatin sections just described it is usually possible to see the layers without the use of any stain. If one wishes to use stain, a little ruthenium red or dilute iodine solution will help to bring out the lamellate structure. Dry mature fibers held firmly in a split cork mounted in a vise were cut by hand. These were used for comparison with the mature fibers taken from unopened bolls.

One of the objects of this study was to examine and measure the fibers under as nearly normal conditions as possible. Kerr (39, p. 229) found it impossible to see the lamellae in the unswollen cotton fibers. Osborne observed (47, p. 287) that in untreated cross sections of cotton fibers, the "growth rings" are too closely packed to be resolved even with the highest numerical aperture and magnifying power although in a footnote he added that an untreated cross section of a fuzz hair showed a few ill-defined growth layers.

By using critical illumination and cutting down the intensity of illumination, it is possible to see the lamellae of fibers from unopened bolls without using either stains or swelling reagents. The measurements were made with a 20 X ocular and 46 X objective, 4 mm., N.A. 0.95, which produce a magnification of about 920 diameters. The spaces upon the ocular micrometer, calibrated by the usual method with a stage micrometer, showed each ocular micrometer space to measure  $2.35\ \mu$  from center to center of adjacent lines with this combination of lenses. Some studies in polarized light were also made at 900 diameters. Most of the photographic records of cross sections were made at these two magnifications. A few photographic records were made at 450 or 460 diameters. Some of these were later enlarged. Greater depth of focus, and a better idea of contour can be obtained by this method (Fig. 2 A).

#### DEVELOPMENT OF THE COTTON FIBER IN DAYLIGHT

The early stages of fiber growth from the day of flowering to the age of 20 days have already been described by Farr (30) for this same genetically pure strain of Super Seven. The very young stages can be seen readily by mounting a piece of the seed coat with developing fibers in water only. These young fibers contain chains of cellulose particles in the wall as well as numerous cellulose particles in the lumen of the cell (Fig. 2 A). The chains of particles form fibrils, and the fibrils form sheets or lamellae which build up the very complex mature fiber wall. Reissek (50) noted granules in the lumen of fibers, and De Mosenthal (26) reported minute spherical granules of nearly uniform size (about  $1\ \mu$ ). New fibers may be initiated for a week or more (30) over the surface of the expanding seed coat. The use of polarized light helps to show the orientation of the cellulose particles in the lamellae and the intricate way in which the fibrils form spirals in the wall.

*Variation.* There is considerable variation in the fiber length of any one



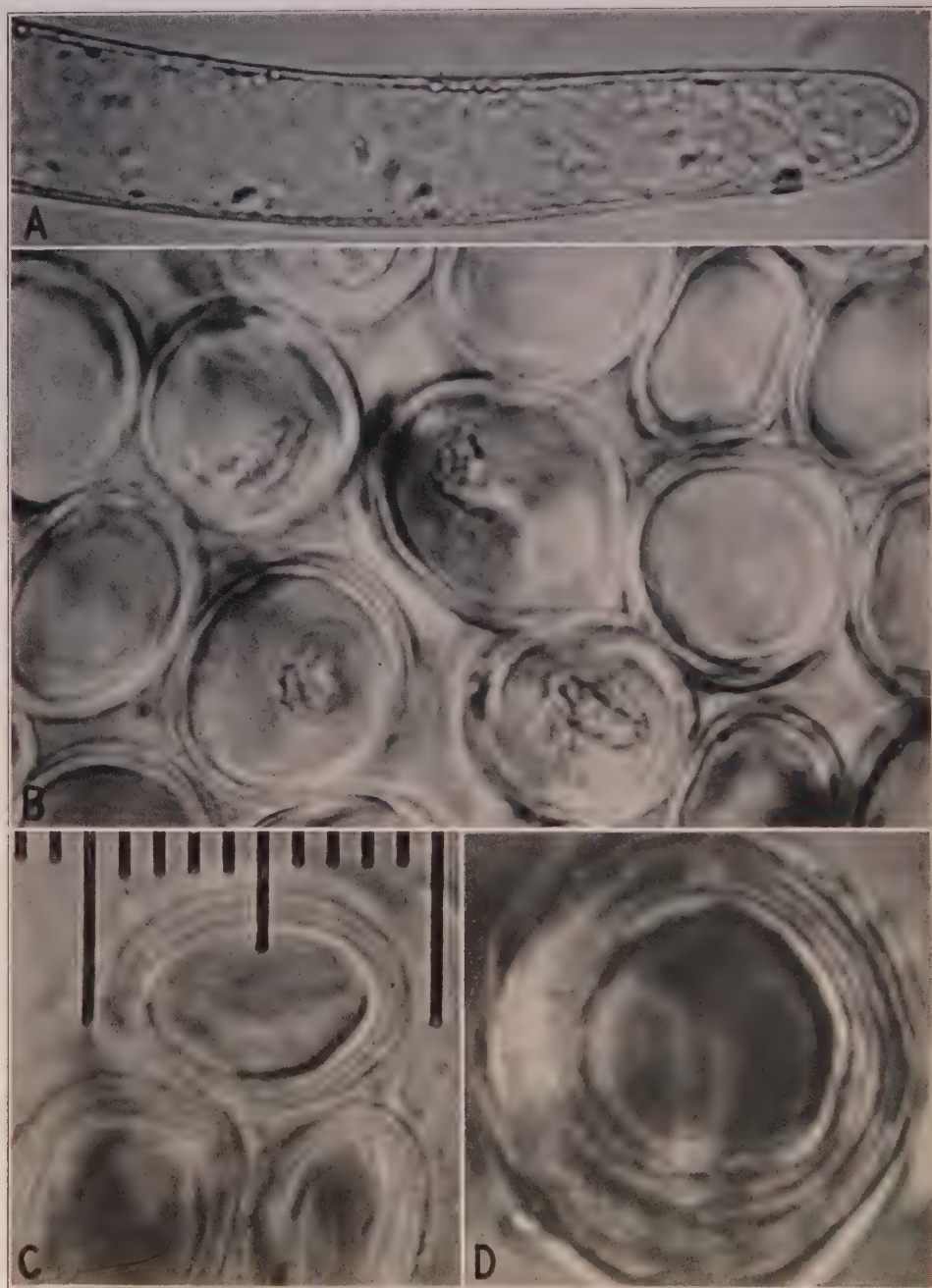


FIGURE 2. (For description see legend on opposite page.)



seed as has already been shown by Richmond and Fulton (51). Sturkie (57), using Mexican Big Boll, found that lack of soil moisture during the first 42 days after flowering could shorten the normal fiber length of 28.6 mm. (one and one-eighth inches) by 3 mm. or one-eighth inch. Armstrong and Bennett (7), using variety Super Seven from the same source as Farr (30), found variations in length of fiber and indications that elongation had not ceased by the 30th day.

Great variation is also found in the diameter of cotton fibers and in the thickness of the walls. This depends upon the age of the fiber and the point on the fiber at which the cross section or measurement is made. Tip as used here refers to cross sections cut from the upper third of the bundle of fibers. Base refers to those cut from the lower third near the seed coat. Middle includes the third in the center. Figure 2 B shows cross sections of the base of 32-day fibers photographed at a magnification of 460 diameters, and then enlarged to 1035 diameters. These fibers were still embedded in gelatin and show variations in size. Masses of cellulose particles are still visible in the lumen of some of the fibers while others were either empty, or the section happened to be cut at a level where no particles were present. Most of these fibers had two or three lamellae. Figure 2 C shows cross sections of the tips of three fibers from the same seed. These were photographed at 920 and enlarged to 1840 diameters. The ocular micrometer scale is in position to measure the long diameter of the upper cross section which had three lamellae.

There is usually a difference in the number of lamellae in the base and in the tip of the fibers from the same seed. Although the 32-day sample illustrated in Figure 2 B and C were about the same in base and tip, the difference in number is marked in the older fibers. More lamellae are found in the more mature fibers as in Figure 2 D of the base of a 45-day sample which had six lamellae. The largest number of lamellae are found at the base of the oldest fibers near the seed coat.

The largest and smallest diameter of the fiber indicates the shape of the cross section. If nearly equal, the cross section approaches a circle. If one measurement is much longer than the other, the shape of the section is an ellipse and the fiber is much flattened. The diameters of the lumen and the thickest and the thinnest parts of the wall were also measured. The two measurements of the wall thickness were averaged. The number of lamellae was counted. From these last two figures the average width of the wall was computed in microns. On most samples of different ages, ten or more cross sections of typical fibers were measured, and this number averaged.

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FIGURE 2. Cotton fibers grown in daylight. Lamellae about  $1\ \mu$  wide. A. 4 days, 1 lamella with cellulose particles ( $\times 575$ , enlarged to 1150). B. 32-day cross section of bases ( $\times 460$ , enlarged to 1035). C. 32-day tips ( $\times 920$ , enlarged to 1840). D. 45-day base ( $\times 1125$ ).

About 10 to 12 lamellae were the largest numbers counted in any of the cotton fibers grown in the greenhouse. The measurements are presented in Table I, sections A, B, C, and D. The trend, with age, of fiber wall thickness, number of lamellae, and thickness of lamellae is shown for both tips

TABLE I  
CROSS SECTIONS OF COTTON FIBERS, VAR. SUPER SEVEN; MEASUREMENTS IN  $\mu$

Age in days	Diameter of fibers		Diameter of lumen		Width of wall		Av. width of wall	No. of lamel- lae	Av. width of lamel- lae in $\mu$ (com- puted)
	Long	Short	Long	Short	Long	Short			
A. Tips of cotton fibers—paraffin section $10\mu$ thick—av. of 10 measurements									
10	28.08	20.56	22.90	16.80	2.11	1.41	1.76	1.20	1.46
B. Tips of cotton fibers—gelatin hand section—av. of 50 measurements									
23	25.60	12.12	22.15	8.95	1.69	1.64	1.66	1.43	1.16
28	27.14	16.28	21.35	11.77	2.79	2.16	2.47	1.99	1.24
33	25.64	17.32	17.46	10.82	4.77	3.76	4.26	3.78	1.13
38	26.84	22.77	16.52	13.25	5.66	4.60	5.13	4.76	1.08
43	26.18	18.47	15.58	8.13	5.88	4.86	5.37	5.24	1.03
48	28.27	19.13	15.04	7.64	6.29	5.26	5.78	5.17	1.12
53	27.40	16.94	13.77	4.65	6.58	5.64	6.11	5.39	1.13
58	26.52	16.70	13.60	4.16	6.16	5.33	5.74	5.44	1.06
C. Bases of cotton fibers—paraffin section $10\mu$ thick—av. of 10 measurements									
5	26.09	19.27	21.15	15.51	2.76	1.64	2.20	1.45	1.52
9	38.43	23.03	22.09	15.27	4.11	2.58	3.34	2.40	1.39
10	23.71	17.62	16.80	11.39	3.40	2.23	2.82	1.55	1.82
11	25.26	14.57	19.97	10.10	3.70	1.52	2.11	1.45	1.46
14	23.50	14.57	19.27	10.92	2.11	1.46	1.79	1.35	1.33
15	29.96	18.21	24.44	13.39	2.58	1.41	1.99	1.50	1.33
20	27.37	18.68	22.79	14.33	2.05	1.41	1.73	1.25	1.38
D. Bases of cotton fibers—gelatin hand section—av. of 50 measurements									
28	26.81	18.39	20.79	13.08	2.93	2.74	2.84	2.38	1.19
33	26.55	21.92	18.68	13.72	4.58	3.83	4.20	4.01	1.05
38	25.60	20.28	15.88	10.76	5.47	4.44	4.95	4.84	1.02
43	28.11	21.80	15.52	9.47	7.31	6.20	6.63	6.34	1.05
48	28.67	19.95	15.22	6.72	7.26	5.94	6.60	6.64	0.99
53	25.59	18.49	11.16	4.04	7.35	6.08	6.72	6.38	1.05
58	27.24	19.57	12.99	5.90	7.86	7.01	7.43	7.16	1.04

and bases in Figure 3. These curves were calculated by the methods of least squares from the original data which are summarized in sections B and D of Table I. They show that the thickness of the lamellae at the tips of the fibers is about  $1.10\mu$  and at the bases is about  $1.03\mu$ . The curves also show that the fiber wall at the base is thicker and has more lamellae than at the tip. These data contain no evidence to support Balls' assumption that

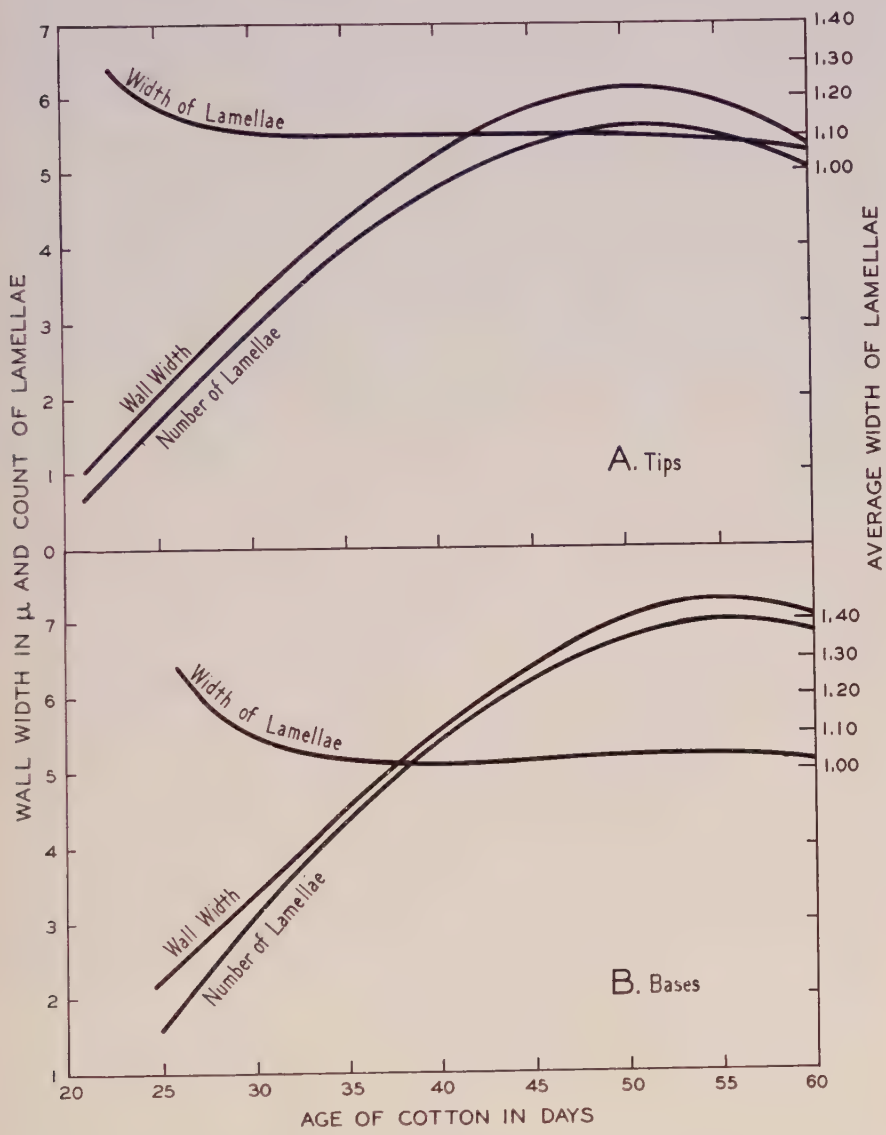


FIGURE 3. Wall width, number of lamellae, and width of lamellae for tips (A) and bases (B) of cotton fibers during development.

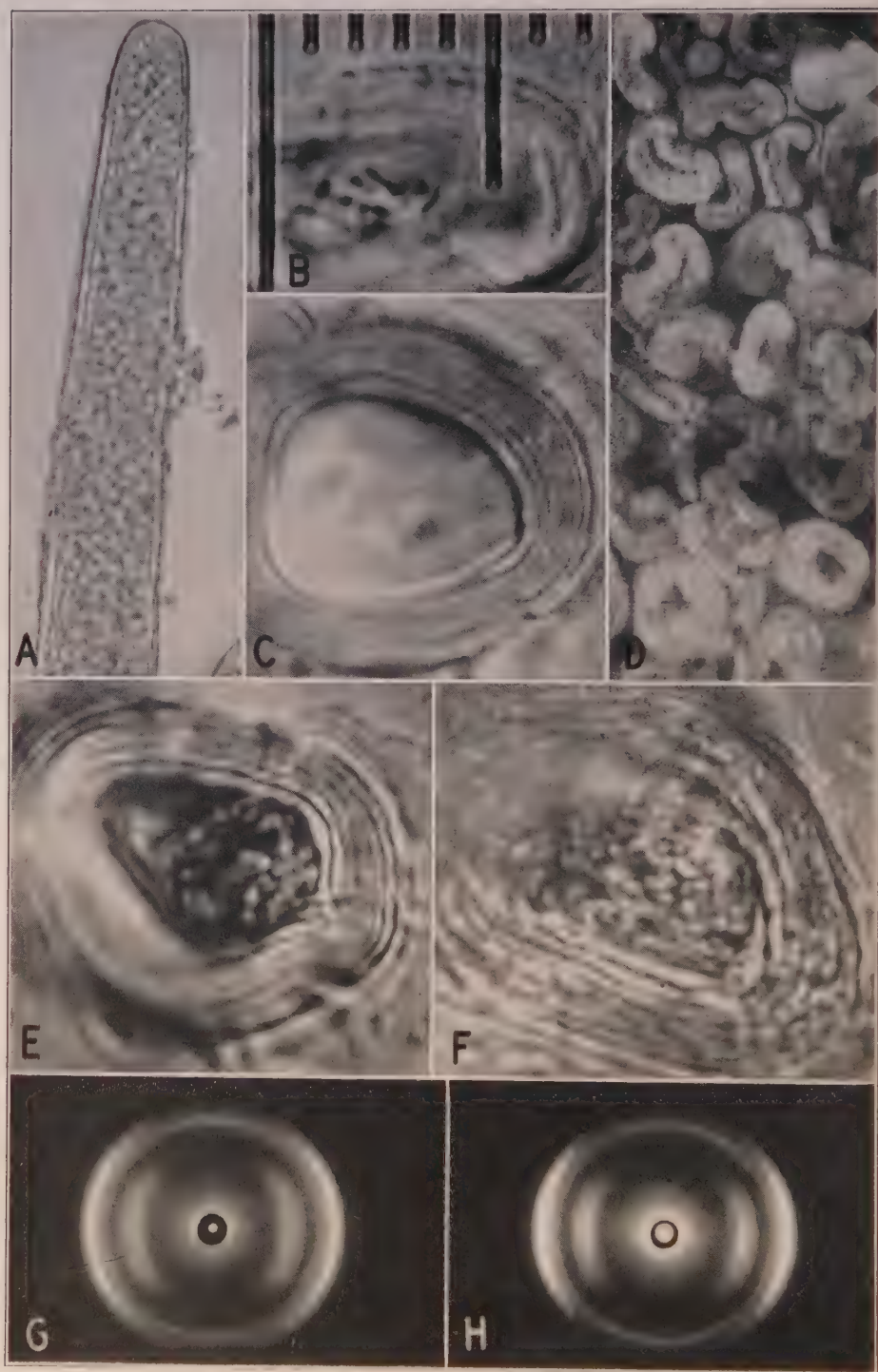


FIGURE 4. (For description see legend on opposite page.)



daily growth rings are formed (11). It requires about 50 to 60 days to develop Super Seven cotton to maturity when grown in the greenhouse. In winter, during the short days, this time is usually increased to 70 to 75 days. In continuous light the bolls matured in 49 to 65 days.

This indicates, as has been microscopically verified repeatedly during this study, that each lamella is made up of a single sheet or layer of cellulose particles held together by a colloidal cementing material. The ellipsoidal particles are arranged end to end in fibrils. The fibrils may have different angles in relation to the longitudinal axis of the fiber. In adjacent fibrils and in adjacent lamellae, the rows of particles are usually staggered so that the widest diameter of one particle fits into the space left between the end-to-end position of the two adjacent particles on either side. This visible microscopic structure which has been observed repeatedly, not only in cotton fibers but also in many other plant membranes (16), makes for flexibility and compactness of the lamellae in the membrane.

#### DEVELOPMENT IN CONTINUOUS LIGHT

Cotton plants in the early cotyledon stage or just breaking the seed coat were transferred from the greenhouse to the constant light room. Bolls were ripened in light from a sodium vapor lamp, and also with a sodium vapor lamp plus two hours' daily additional light from a capillary mercury arc lamp. During the second growing season an air-conditioning unit maintained the temperature at 60° to 63° F. Better growth and a larger number of bolls resulted from this relatively constant temperature.

Although the mature fiber walls are somewhat thinner than those of the greenhouse plants, these continuous light fibers showed lamellate structure similar to that of the plants grown in daylight. No difference in structure could be found except in the slightly reduced number of lamellae in mature fibers as compared with controls grown in the greenhouse. Fibers grown in continuous light were examined from time to time during development. From the earliest stages the fibers contained cellulose particles. Figure 4 A shows a 15-day fiber with one lamella already formed and a second forming just below the tip. The lumen of the cell is filled with cellulose particles—the building units of which the fibrils and lamellae are constructed. Figure 4 B has six of the cellulose particles in a cross section photographed against the micrometer scale, each space of which, measured from center to center of adjacent lines, is equal to  $2.35\ \mu$ . It will be seen that the particles are approximately a micron in width. In Figure 4, C, E,

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FIGURE 4. Cotton fibers grown in constant light. Lamellae about  $1\ \mu$  wide. A. 15 days, 1 lamella, 2nd forming ( $\times 920$ ). B. 49-day cross section; 6 cellulose particles with micrometer scale (1 space equals  $2.35\ \mu$  measured from center to center of adjacent lines). C, E, F. 49-day cross sections at middle. D. 60-day dry, cut in cork ( $\times 460$ , enlarged to 890). G. X-ray diffraction pattern of fibers matured in constant light. H. In daylight.

TABLE II

CROSS SECTIONS OF COTTON FIBERS, VAR. SUPER SEVEN, GROWN IN CONTINUOUS LIGHT; AVERAGES OF 10 MEASUREMENTS EACH IN  $\mu$ 

Age	Treatment			Diameter of fibers		Diameter of lumen		Width of wall		Av. width of lamellae in $\mu$ (computed)	No. of lamellae	Av. width of lamellae in $\mu$ (computed)
	Light	Medium	Sample	Long	Short	Long	Short	Long	Short			
Mature	Na 24 hrs.	Gelatin	Tip	21.63	11.16	10.22	1.41	5.52	4.70	4.80	1.08	
"	"	"	Base	27.73	12.80	13.39	1.17	6.09	5.52	5.70	1.07	
"	Na 24 hrs. Hg 2 hrs.	Cork	Middle	21.38	8.93	12.92	1.46	4.81	3.47	3.90	1.06	
"	"	Gelatin	Tip	26.20	11.63	12.57	1.64	5.64	4.58	4.60	1.11	
49 days	"	"	Base	23.50	22.33	11.16	1.17	5.75	4.58	5.00	1.03	
"	"	"	Middle	23.30	14.10	14.57	6.11	4.81	3.99	4.40	1.00	
60 days	"	"	Middle	21.03	13.04	12.33	4.46	4.46	3.87	4.00	1.04	
"	"	Cork	Middle	27.02	10.69	16.92	0.70	5.17	4.23	4.75	0.98	
59 days	Na 24 hrs. Hg 2 hrs.	Gelatin	Tip	23.38	12.80	10.45	Slit	6.31	5.52	5.50	1.07	
"	"	"	Base	24.32	13.39	11.75	"	6.31	5.57	5.40	1.08	
60 days	"	"	Tip	22.32	18.86	10.45	"	6.34	5.64	5.30	1.12	
"	"	"	Base	25.14	13.39	12.69	0.47	6.31	5.52	5.40	1.09	
65 days	"	"	Tip	21.38	10.34	11.51	Slit	5.28	4.81	5.05	1.17	
"	"	"	Base	24.44	11.16	15.62	0.82	4.93	4.93	4.30	1.14	
"	"	"	Tip	21.50	8.22	13.74	0.35	3.81	3.81	3.17	0.97	
"	"	"	Base	27.02	12.57	15.39	1.29	5.64	5.05	5.05	1.05	
Total											Average	17.06
												1.06 $\mu$

and F are also cross sections of the middle of fibers grown in continuous light which were mature at 49 days. Figure 4 F shows cellulose particles in the lumen, and some particles are still visible in the lamellae. The lamellae are just as distinct as in fibers grown in daylight in the greenhouse. Figure 4 D, 60-day dry fibers cut in cork, illustrates thin- and thick-walled mature fibers. The thin-walled fibers often collapse and form curved cross sections. It is much more difficult to see lamellae in dried fibers than in those kept moist in gelatin. Table II shows typical measurements on mature fibers grown in continuous light. It will be seen that these cotton fibers grown in continuous light have lamellae whose average width for the 160 fibers measured was  $1.06 \mu$ . The time of development of the bolls in continuous light has been 49 to 65 days. The number of lamellae found in mature fibers grown in continuous light was about five to seven (Fig. 4 C).

*X-ray analysis.* X-ray diagrams of mature cotton fibers grown under continuous light and under daylight in the greenhouse are shown in Figure 4 G and H. As pointed out by Sisson (55), the type of X-ray diagram given by these two samples does not indicate whether lamellae are present. The pattern does imply that the cell wall contains separated crystalline units having a spiral orientation with reference to the fiber axis. The most logical interpretation of the X-ray requirements is met by a cell wall built up of spirally wound fibrils which are arranged in lamellae. Although the two samples give the same type of X-ray diagram, which implies the same general type of structure, the patterns differ in two details. First, H, the daylight sample grown in the greenhouse, has the diffraction rings concentrated into sharper arcs. This indicates that the crystalline cellulose is more definitely oriented (55, p. 56) in the cell wall than in G, the continuous light sample. Second, H shows slightly less scattered radiation (fogging or background) and a better resolution of the cellulose diffraction lines than in continuous light, G. This latter difference is less distinct than the orientation, but is evident in the original negative. On the basis of Sisson's experiments (55), this may be interpreted as indicating less of the non-cellulosic material in fibers grown in the greenhouse, H. It is probable that this difference in the X-ray diagrams may be explained by the microscopic evidence which shows that the cotton fibers grown in continuous light have thinner walls and fewer lamellae than those grown in daylight in the greenhouse. The crystalline cellulose of both is of the same nature, but since the relative amount of crystalline cellulose to non-cellulosic material is slightly different, one would infer that this is due to the fact that there are more lamellae and consequently a slightly higher percentage of cellulose in the daylight sample grown in the greenhouse, G.

#### DISCUSSION

Balls and Hancock (14) seem to have been the first to grow cotton under continuous light. Anderson, in a personal communication to Seifriz

(54, p. 132) reported stratification in a plant which had received continuous (artificial) illumination. In 1935, Anderson and Moore (4) reported that fibers grown under constant artificial illumination possess stratification (layers or lamellae) similar to that characteristic of the cell walls of fibers grown under intermittent conditions. Subsequently Anderson in 1936 (2) stated that fibers grown under constant artificial illumination showed no lamellation. Anderson and Moore in 1936 (5) reported no evidence of growth rings. Anderson and Moore, using *Gossypium hirsutum* var. Mexican Big Boll, came to the conclusion that (6, p. 507) cotton fibers grown under continuous artificial light have no growth rings in their walls. Their cross sections were sectioned, swollen, and stained after the method described by Kerr (39). As previously indicated by Denham (27) and Balls (11), this cuprammonium hydroxide method is a very drastic one and causes distortion. The users of the cuprammonium hydroxide method have assumed that all components of cellulose membranes swell uniformly in this reagent, although they offer no measurements or evidence to support their assumption. As Farr (31, 32) and Compton (24) have shown, cuprammonium hydroxide has a differential effect upon the two main components of cellulose membranes. The colloidal cementing material around the cellulose particles and between the lamellae swells, but the size of the cellulose particles themselves is unaltered. When pressure is used on cross sections of cotton fibers swollen in cuprammonium hydroxide—the method used by Bailey and Kerr (10), Kerr (39), and Anderson and Moore (6)—the lamellae are greatly distorted, and the cellulose particles are pushed out of their normal position. With such a procedure, an accurate count of the lamellae is difficult if not impossible. The appearance of lamellae as in their Figure 5 may be the result (6, p. 506), but it is not at all a fair representation of the actual structure of the cotton fiber. Moore and Anderson's latest paper on "cotton fibers without 'growth rings'" has a figure of transverse sections of field-grown and continuous light fibers of Mexican 128-6 variety (45, p. 326, Fig. 1). The magnification was not indicated, but it must be relatively low. Little of the structure was visible except that there were more thin-walled fibers in the continuous light sample.

Tiffany (59) found that continuous illumination did not affect the lamellae in algae, where light might be expected to have a more direct effect than within a cotton boll.

Cotton fibers of *G. hirsutum* var. Super Seven and Acala when grown under continuous light according to the conditions of Arthur and Harvill (8) from the early cotyledon stage to maturity show no differences from those grown in daylight in the greenhouse except in the thickness of the fiber wall. Cellulose particles form fibrils and lamellae the same as in any fiber grown in daylight in the greenhouse. The lamellae are about  $1\ \mu$  in thickness. The only difference visible microscopically is a higher percent-



age of thin-walled fibers with correspondingly smaller average number of lamellae than in fibers grown in daylight in either greenhouse or field.

*Diameter.* Measurements of the diameter of cotton fibers by Peirce (48) showed a range of 15.2 to 21.1  $\mu$  in diameter in six varieties. Calvert and Harland (21) found a variation from 11.9  $\mu$  in Sea Island to 20.3  $\mu$  in Peruvian. They measured from 400 to 600 mercerized hairs each of 35 different cottons. Brown, Selim, and Balls (19) found a diameter range of 5 to 19  $\mu$  in 200 cotton hairs.

Pope (49) measured diameters of three strains of cotton grown in Arkansas. He used ginned samples wet in aqueous gentian violet, and measured with a filar micrometer (standardized by a stage micrometer) and a euscope. Five hundred measurements were made for each strain. His Table I (49, p. 963) shows a range of classes from 7.1  $\mu$  to 8  $\mu$  to 36.1  $\mu$  to 37  $\mu$ .

Moore (43) in a study of seven strains of American upland cotton (*Gossypium hirsutum* L.) compared 400 mercerized fibers with 600 uncollapsed fibers of each strain. The mercerized had been treated with 18 per cent NaOH. The uncollapsed were fixed in acetic-formalin-alcohol before being embedded in gelatin. Very "concentrated gelatin" at 60° C. was used, later dehydrated with 95 per cent ethyl alcohol and 5 per cent commercial formaldehyde (43, p. 607). Cross sections were made on a sliding microtome and mounted in glycerin jelly. This was a modification of the Clegg and Harland method. A microprojection method was used to secure tracings and the drawings were measured. For the uncollapsed samples the diameter range was 23.18  $\mu$  to 26.09  $\mu$  with an average of 24.731  $\mu$ . The mercerized samples ranged from 14.47 to 17.20 and averaged 15.886  $\mu$ . Fiber diameter is reported to be inherited and a genetically stable character.

Another report by Moore on the same seven strains (44), using mercerized 200-fiber samples, gave a range of 16.0 to 17.7  $\mu$  for the 1932 crop and 16.4 to 18.0  $\mu$  for the 1934 crop. The mercerization was carried out in the same way and a microprojection apparatus was used to measure the width of the mid-portion of 200 fibers of each sample.

*Wall thickness.* Herzog (37, p. 1098) has a table of measurements on two hairs with lengths of 2120  $\mu$  and 2526  $\mu$  from base to tip. He found that wall thickness ranged from 6.9  $\mu$  to 12.1  $\mu$ . Morton (46) found a wall thickness varying from 3.25 to 7.70  $\mu$  in numerous mill samples. Clegg (22) measured 200 mature fibers of each of 14 varieties of cotton and found a range of wall thickness from 3.0  $\mu$  to 8.5  $\mu$ . Karrer and T. L. W. Bailey, Jr. (38) reported a wall thickness of from 0.35 to 15.5  $\mu$ . Their method was to take photographs of cross sections magnified 250 times and enlarged to 1000 diameters. Measurements were made on the enlarged photographs, not on the actual cross sections.

Hawkins and Serviss (35), using paraffin material sectioned and stained

with gentian violet, reported on development of wall thickness in Pima and Acala. Their figures range from  $0.3\ \mu$  for six days to  $3.20\ \mu$  for 75 days.

Sakostchikoff and Korscheniovsky (52) gave measurements of wall thickness of fresh hairs ranging from  $0.92$  for 23 days to  $6.34\ \mu$  for 68 days. These figures fall within the probable range of fiber measurements as indicated in the present study.

#### SUMMARY

1. The development of cellulose membranes from the day of flowering to maturity has been studied in the epidermal hairs of the seed coat of *Gossypium hirsutum* L. in a genetically pure line of variety Super Seven. The cotton fiber contains cellulose particles which are laid down in successive lamellae, thus gradually increasing the thickness of the fiber wall.

2. There is great variability of wall thickness in the fibers on the same seed. The number of lamellae at base and tip varies in fibers on the same seed, the larger number of lamellae being at the base of the older fibers. No evidence has been found to correlate these lamellae with Balls' so-called *daily* "growth-rings."

3. The lamellae are of remarkably uniform thickness being about 1 micron thick in the cotton fiber, whether grown under daylight in the greenhouse or in continuous light. The microscopical and microchemical evidence indicates that each lamella is made up of a single layer of cellulose particles held together by a colloidal cementing material.

4. X-ray diffraction patterns of cotton fibers grown in continuous light and in daylight in the greenhouse indicate the same general type of structure with only slight differences in orientation and amounts of cellulose.

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# SOME EFFECTS OF TREATMENT OF NON-DORMANT SEEDS WITH CERTAIN GROWTH SUBSTANCES

LELA V. BARTON

## INTRODUCTION

Several reports have appeared in recent years as to the effect of certain synthetic growth substances on seed germination and on the further development of seedlings produced from seeds so treated. The present paper is a report of results obtained using many different kinds of seeds which germinate promptly. Germination and growth effects will be considered.

Both liquid and dust treatments were given. In the liquid treatments, seeds were soaked for various lengths of time in the solutions after which the liquid was drained off and the unwashed seeds placed under germination conditions. For dust treatments the seeds were mixed thoroughly with an excess of the dust after which the excess was sieved off and the seeds placed to germinate. Because of the different types of experimental data to be considered, the specific methods used as well as the results obtained will be described in the individual units of the paper.

## EXPERIMENTAL RESULTS

The seeds used in these tests were:

<i>Scientific name</i>	<i>Common name and variety</i>
<i>Allium cepa</i> L.	Onion—Crystal Wax Bermuda
<i>Antirrhinum majus</i> L.	Snapdragon—Day Dream
<i>Avena</i> sp.	Oats—Storm King
<i>Beta vulgaris</i> L.	Beet—Crimson Globe
<i>Brassica rapa</i> L.	Turnip—Purple Top White Globe
<i>Daucus carota</i> L. var. <i>sativa</i> DC.	Carrot—Chatenay or Model
<i>Delphinium ajacis</i> L.	Larkspur—Giant Hyacinth-Flowered
<i>Festuca pratensis</i> Huds.	Meadow fescue
<i>Glycine max</i> Merr.	Soybean—Cayuga
<i>Lactuca sativa</i> L.	Lettuce—Iceberg
<i>Lolium perenne</i> L.	English rye grass
<i>Lycopersicon esculentum</i> Mill.	Tomato—Pritchard or Scarlet Topper
<i>Matthiola incana</i> R. Br.	Stock—Double, Early, Giant, Imperial
<i>Paeonia suffruticosa</i> Andr.	Tree peony
<i>Phytolacca decandra</i> L.	Pigeon berry
<i>Pisum sativum</i> L.	Garden peas—Extra Early
<i>Poa pratensis</i> L.	Kentucky Blue grass

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<i>Raphanus sativus</i> L.	Radish—Crimson Giant Globe
<i>Raphanus sativus</i> L.	Radish—French Breakfast
<i>Secale cereale</i> L.	Spring rye
<i>Solanum melongena</i> L. var. <i>esculentum</i> Nees.	Eggplant—Kilgore's Bred-Rite Mana- tee Special
<i>Tagetes erecta</i> L.	Marigold—Lemon Ball
<i>Tagetes erecta</i> L.	Marigold—Orange Ball
<i>Tagetes patula</i> L.	Marigold—Dwarf Double French
<i>Triticum</i> sp.	Egyptian wheat
<i>Triticum</i> sp.	Marquis wheat
<i>Tropaeolum majus</i> L.	Nasturtium — Beauty (dwarf single- flowering)
<i>Zinnia</i> sp.	Zinnia—Crimson Gem Pompon
<i>Zinnia</i> sp.	Zinnia — Giant Dahlia-flowered Exqui- site

## EFFECT ON GERMINATION

Very few workers have been concerned with the effects of growth substances on germination alone but several reports have included these effects with others.

Davies, Atkins, and Hudson (5) germinated seeds of oats, mustard, and cress under sterile conditions in Pfeffer solution containing compounds at a concentration of 1 part in 10,000 and found both germination and subsequent growth retarded by  $\beta$ -indolyl propionic and  $\beta$ -indolyl acetic acid. The following year, 1937, the same workers (6) reported further experiments using the same seeds but testing more indole derivatives with the same general results obtained in the previous tests.

Three papers published in 1938 dealt with germination effects of this same group of substances. An article in the Annual Report of the Maryland Agricultural Experiment Station (16) reported the treatment of seeds with solutions or dust preparations of growth regulators and found an increase in the percentage germination of treated seeds. Neither the kinds of seeds, nor the manner of treatment, was described and no data were presented.

Čajlachjan and Ždanova (3), using seeds of summer and winter wheat, oats, millet, summer vetch, hemp, white mustard, perilla, flax, and peas, soaked them for 24 hours in aqueous solutions of  $\beta$ -indolyl acetic acid in concentrations of 10, 25, and 50 mg./100 cc. They found that high concentrations stopped sprouting in all cases, and no stimulation of germination was obtained.

Shibuya (19) germinated rice and flax seeds on filter paper wet with 8 cc. of 40, 20, 10, 1, 0.1, 0.05, and 0.01 mg./100 cc. of  $\beta$ -indole-acetic acid and found no differences in the germination of rice seeds but in the case of



flax he obtained retarded and reduced germination which increased with increased auxin concentration.

Herbst (12) found that heteroauxin did not affect tomato seed germination enough to make pretreatment worth while. Such pretreatment he found influenced the growth of the young plants, but the stimulation was not significantly greater than with seeds germinated in garden soil.

It will be noted that all of these authors, with the exception of the report from the Maryland Agricultural Experiment Station, which gave no data, are agreed that germination is not stimulated by treatment of the seeds with growth substances and that retardation or reduction in germination results from treatment with high concentrations.

Experiments to be presented below show the effect on germination of both liquid and dust treatments of several kinds of seeds with growth substances.

*Experiment 1.* In October, 1937, an experiment was begun to show the effects of different concentrations of solutions of indoleacetic, naphthaleneacetic, and indolebutyric acids on the germination of seeds of tomato, wheat, and *Phytolacca decandra*. Since *Phytolacca* seeds had been shown by previous experiments to possess hard seed coats some of these seeds were treated after the coats were broken. Concentrations of 316.0, 31.6, 3.16, and 0.316 mg./l. were used for each acid. Seeds were soaked in the solutions for 22 hours at room temperature after which the solutions were drained off and the unwashed seeds placed in ovens at controlled temperatures for germination. Temperatures used for germination were those shown by previous experiments to be satisfactory. Constant temperatures of 20° and 25° C. were used for wheat and tomato respectively while a daily alternating temperature of 5° to 30° C. was used for *Phytolacca*.

No stimulation of germination was obtained in any case. Complete inhibition of germination was obtained when the seeds of *Phytolacca*, the coats of which had been broken, were subsequently treated with naphthaleneacetic acid in a concentration of 316.0 mg./l. In no other case was either inhibition or reduction in germination noted. Other effects, however, were evident. All concentrations of naphthaleneacetic acid caused some thickenings of the roots of the tomato seedlings and increased the number of root hairs. The stronger concentrations stunted growth markedly and caused many proliferations. Treatment with 316.0 mg./l. of naphthaleneacetic acid also produced proliferations on roots of wheat seedlings, but seeds treated with the weaker solutions produced normal roots. *Phytolacca* seedlings were all normal in appearance if the seeds germinated at all.

Solutions of indolebutyric acid in all concentrations employed caused pinkish enlarged root tips and the production of many root hairs on all tomato seedlings, and the strongest concentration, 316.0 mg./l., caused proliferations on the roots of wheat.

Treatments of tomato seeds with all concentrations of indoleacetic acid resulted in apparently normal seedlings but the strongest concentration, 316.0 mg./l., also caused proliferations of wheat roots.

These plants were not grown in soil but were germinated on moist filter paper at controlled temperatures and were allowed to grow as long as possible for observation.

This preliminary test, then, indicated that injury from too strong concentrations of these substances could be easily demonstrated, whereas it was not possible to demonstrate any stimulation under the conditions of this experiment. Furthermore, it was shown that solutions of naphthaleneacetic acid and indolebutyric acid were more toxic to seeds than corresponding concentrations of indoleacetic acid and that naphthaleneacetic acid was most toxic to seeds the coats of which had been broken.

*Experiment 2.* In September, 1938, a new experiment was begun with seeds of Marquis wheat and tomato with the object of performing germination tests both on filter paper in ovens at controlled temperatures and in soil in the greenhouse. In the latter case it was possible to transplant any seedlings obtained and trace the possible effect of seed treatment on the further growth of the seedlings.

Duplicate lots of seeds were soaked in both dark and light at room temperature for 24 hours prior to germination tests. For these tests potassium  $\beta$ -indoleacetate and potassium  $\alpha$ -naphthaleneacetate in concentrations of 320.0, 106.6, 35.5, 11.8, and 3.7 mg./l. were used. Water controls were also used. Two commercial powders, Auxan and Rootone, together with dry controls were also used.

These tests indicated no differences in the germination of seeds of either wheat or tomato whether the soaking was in dark or light. Wheat seeds were injured by soaking in solutions of both potassium  $\beta$ -indoleacetate and potassium  $\alpha$ -naphthaleneacetate in all concentrations. This was partially a soaking effect since the water control showed a reduction of approximately 50 per cent germination as compared with the dry control. However, a further harmful effect was evident when concentrations of 320.0 mg./l. and 106.6 mg./l. of potassium  $\alpha$ -naphthaleneacetate were used. Only occasional seedlings were produced after the former treatment. On the other hand, the germination of tomato seeds was very little affected by any of the concentrations of potassium  $\beta$ -indoleacetate, but 320.0 mg./l. of potassium  $\alpha$ -naphthaleneacetate reduced germination to about one-third that of the controls. Germination was reduced to a smaller degree by a concentration of 106.6 mg./l. of potassium  $\alpha$ -naphthaleneacetate. The lower concentrations were seemingly without effect. The comparative effects of the various treatments are shown graphically in Figure 1 which was taken about one month after treatment and planting of the seeds. Here, again, the harmful effects on germination of soaking in the stronger concentrations of potassium  $\alpha$ -naphthaleneacetate are evident.

*Experiment 3.* A repetition in a second series testing tomato and wheat seeds produced similar results.

The appearance of seedlings from treated seeds, together with further growth of the plants from these seeds, will be reported below.

*Experiment 4.* It was then thought desirable to try a variety of seeds to see whether the germination of some one form might not be found to benefit by the application of growth substances either in liquid or dust form to the seeds. Seeds of eggplant, larkspur, lettuce, French marigold, nasturtium, onion, garden pea, radish, snapdragon, stock, tomato, and zinnia were soaked in solutions of potassium  $\alpha$ -naphthaleneacetate of 320.0, 106.6, 35.5, 11.8, 3.7, and 1.2 mg./l. and water for 24 hours at room temperature after which duplicate lots were placed to germinate on filter paper or in moist granulated peat moss in ovens at controlled temperatures. Duplicate lots from each treatment were also planted in a greenhouse in soil composed of leaf mold, humus, and sand with complete fertilizer added. The greenhouse temperature used was approximately 21° C. for all seeds except larkspur which were placed in a 15° C. greenhouse.

The number of seeds in each sample is shown in column 1 of Table I. For example, the oven test of onion seeds was made at a constant temperature of 25° C. and duplicates of 100 seeds each were used for each test, or 200 seeds for the oven test and 200 seeds for the greenhouse test. Filter paper was used for all oven tests except peas and nasturtium in which cases moist granulated peat moss was used.

Rootone and a dust containing 5 mg./g. of growth substance, made by diluting a Merck powder preparation of 35 mg./g. with talc, with dry seeds as controls, were also used.

Resulting germination percentages are shown in Table I. It will be seen that while seeds of different plants varied in their sensitiveness to the stronger solutions none of them was favored by the weaker solutions. French marigold and tomato seeds were most resistant to injury, showing no significant reduction in germination even when soaked in 320.0 mg./l. of potassium  $\alpha$ -naphthaleneacetate. Eggplant, onion, radish, stock, and zinnia were intermediate in their response to the strongest solutions, having their germination reduced by such treatment. Larkspur and lettuce were much more susceptible to injury.

The general trend of results from soil tests agreed with those obtained in the ovens at controlled temperatures although in most cases the actual percentage germination was less in the former case. Nasturtium was an exception to this rule for in this case germination percentages in peat at 15° C. were considerably less than those obtained in soil at 21° C.

When dust-treated seeds are compared with the untreated dry controls, it will be seen that no differences in germination percentages are evident.

The same test as described above was repeated using seeds of Lemon and

TABLE I

GERMINATION PERCENTAGES OBTAINED AFTER TREATMENT OF SEEDS WITH GROWTH SUBSTANCES. FIGURES REPRESENT AVERAGES OF DUPLICATE LOTS

Seed and No. of seeds per duplicate	Green-house or oven (temp. °C.)	Soaked 24 hours in solutions (mg./l.) of potassium $\alpha$ -naphthaleneacetate						Dust treatments			
		320.0	106.6	35.5	11.8	3.7	1.2	Water control	Merck 5 mg./g.	Ro-tone	None
Eggplant, 100	G.H. 25	41	70	81	76	86	85	89	83	81	95
		62	87	84	87	84	90	92	82	75	93
Larkspur, 50	G.H. 15	22	36	44	68	69	60	65	36	32	73
		9	14	65	67	73	71	74	74	68	78
Lettuce, 100	G.H. 20	0	6	25	34	19	17	35	47	46	47
		0	27	43	40	60	67	61	71	76	79
French mari-gold, 50	G.H. 20 to 30*	94	98	99	99	98	96	100	87	98	97
		85	96	97	96	99	92	94	94	95	99
Nasturtium, 20	G.H. 15	30	40	58	48	27	38	53	70	53	65
		0	8	3	13	25	13	3	43	40	35
Onion, 100	G.H. 25	52	39	54	45	58	56	62	66	71	61
		32	53	48	57	44	62	47	74	70	70
Pea, 50	G.H. 20	16	43	74	74	65	81	73	79	84	89
		76	95	95	99	98	97	100	99	100	100
Radish, 100	G.H. 20	33	64	66	73	70	64	76	49	52	72
		67	74	87	85	87	79	83	90	84	87
Snapdragon, 100	G.H. 15	6	23	42	52	49	48	57	57	66	57
		63	81	84	78	91	82	85	89	82	83
Stock, 50	G.H. 20 to 30*	47	62	38	80	82	90	77	89	86	91
		55	87	95	92	97	97	79	93	93	99
Tomato, 100	G.H. 25	84	92	95	86	86	85	93	85	94	93
		90	90	94	93	96	95	98	93	92	100
Zinnia, 25	G.H. 20 to 30*	60	72	72	74	98	80	76	96	80	84
		42	62	78	82	74	78	78	94	94	90
Lemon Ball marigold, 50	G.H.	7	15	17	20	26	24	26	17	20	27
Orange Ball marigold, 50	G.H.	67	73	73	70	72	83	69	71	73	69
Zinnia, 30	G.H.	78	80	88	92	91	90	85	84	88	90

\* Daily alternation.

Orange Ball marigold and zinnia. Here in addition to the dry seeds, talc-dusted seeds were used as controls. These seeds were germinated in soil only but showed the same results as described above (Table I).



The hypocotyl and radicle lengths of some of the seedlings germinated on filter paper and measured three, four, and six days after planting gave qualitative differences which will be reported below.

*Experiment 5.* Still another germination test was begun in March, 1939, using seeds of a number of grains and extending the range of concentration of indolebutyric acid. Merck powder preparations of concentrations of growth substance of 12, 8, 5, and 2 mg./g. as well as Rootone were used. Dry seeds and 325-mesh talc-dusted seeds served as controls. Duplicates of 200 seeds each were used for these tests and all plantings were made in the greenhouse in sod soil with adequate amounts of shredded manure and commercial fertilizer added.

The germination results are shown in Table II. It should be kept in mind that the lawn seed was a mixture, and variation in germination was probably due to error of sampling. It is evident from a study of these data that neither harmful nor stimulatory effects were found as far as germination was concerned. The effect on further growth of these plants will be presented below.

TABLE II

EFFECT OF DUST TREATMENT OF SEEDS ON SEEDLING PRODUCTION IN THE GREENHOUSE.  
FIGURES REPRESENT AVERAGES FROM DUPLICATES OF 200 SEEDS EACH

Seed	Rootone	Merck dusts				Talc	None
		2 mg./g.	5 mg./g.	8 mg./g.	12 mg./g.		
Meadow fescue	92	95	91	92	100	97	96
Perennial rye grass	96	97	98	98	98	97	98
Kentucky Blue grass	57	69	50	66	46	52	58
Stairgreen lawn seed	58	38	28	56	42	43	41
Seed oats	99	98	98	96	98	97	95
Spring rye	78	83	84	82	83	83	82
Soybeans	88	88	80	88	88	87	86
Marquis wheat	79	75	80	77	81	78	78

*Experiment 6.* This experiment was designed to show possible effects of growth substance treatment on seeds under special conditions.

There has been some controversy as to whether seeds which had had their vitality reduced by ageing are not benefited by treatment with such substances. Amlong and Naundorf (1) found that it was possible to increase the germination capacity of older and poorly germinating seeds by soaking for 24 hours in 0.01 N or 0.001 N solutions of indoleacetic acid. They further stated (2) that the germination capacity of 12 kinds of poorly germinating seeds rose as follows when soaked 24 hours in the N/1000 concentration of solutions indicated: indolebutyric acid, 106 per cent; naphthaleneacetic acid, 89 per cent; and heteroauxin 85 per cent.

In view of this possibility of a favorable effect of growth substances on the germination of old seeds, tests were made on six-year-old tomato seeds

which had been dried before storage in a sealed container at room temperature and which still gave a satisfactory germination percentage. Other tomato seeds of the same age with a higher moisture content stored in a sealed container at room temperature and which gave a very low germination percentage (approximately 10 per cent) were also used. Fresh seeds were used as controls. Samples of all lots were soaked for 24 hours at room temperature in concentrations of 320.0, 106.6, 35.5, 11.8, and 3.7 mg./l. of potassium  $\beta$ -indoleacetate and potassium  $\alpha$ -naphthaleneacetate after which duplicates of 100 seeds each were planted on moist filter paper at a constant temperature of 25° C. A similar planting of duplicates was made in soil in the greenhouse. All seeds responded in a similar manner to the treatments. A slight reduction in germination percentage was noted for all three lots on filter paper after treatment with potassium  $\alpha$ -naphthaleneacetate 320.0 mg./l. and a marked decrease in seedling production (from approximately 70 per cent to approximately 7 per cent) was obtained in the greenhouse after such treatment of fresh and old fully-viable seeds. No seedling production in the greenhouse was obtained from the seed lot which was capable of only 10 per cent germination on filter paper.

In short, the germination response of six-year-old tomato seeds of either good- or poor-germination quality to treatment with growth substances was similar to that of fresh seeds under the conditions of this experiment.

This result was to be expected in view of the report of Cholodny (4), who stated that the endosperms of oats, maize, and other grains manufacture growth substance which is then passed on to the embryos and that this growth substance production depends on the weight of the seed and not on its vitality. Nielsen (15) also found that all seeds, both young and old, were rich in growth substance and that, therefore, no relation exists between the amount of growth substance and the germination ability.

That lettuce seed will not germinate to any extent at 30° C. unless pretreated in some way is a well known fact. It was, therefore, of some interest to find whether presoaking in solutions of growth substances would condition the seeds so that germination would take place at this unfavorable temperature. Consequently lettuce seeds were soaked for 24 hours at 20° C., a favorable temperature for lettuce seed germination, in solutions of 320.0, 106.6, 35.5, 11.8, and 3.7 mg./l. of potassium  $\beta$ -indoleacetate and potassium  $\alpha$ -naphthaleneacetate and in water. After this soaking period one-half of the cultures were transferred to 30° C. for germination while one-half were allowed to remain at 20° C.

Presoaking at 20° C. in concentrations of 320.0 and 106.6 mg./l. of both potassium  $\beta$ -indoleacetate and potassium  $\alpha$ -naphthaleneacetate reduced the germination percentage of lettuce when seeds were allowed to re-

main at 20° C. for germination. Lower concentrations permitted approximately the same germination as the water control, which gave 89 per cent. The dry control, which was not presoaked but which was placed on moist filter paper at the same time the soaked seeds were removed from the liquids, gave approximately the same germination as water control but the appearance of the first seedlings from dry seeds was slightly delayed.

When lettuce was placed at 30° C. for germination, no seedlings were produced from seeds presoaked in 320.0 mg./l. of either potassium  $\beta$ -indoleacetate or potassium  $\alpha$ -naphthaleneacetate which had permitted 17 and 7 per cent respectively at 20° C. Very low germination was obtained from other treatments as well, and in no case did the growth substance-treated seeds equal the water-treated for germination at this unfavorable temperature. Treatment with 1.2 mg./l. of potassium  $\alpha$ -naphthaleneacetate was no more effective than with 3.7 and 11.8 mg./l. Dry seeds gave only 1 per cent germination at 30° C.

Under the conditions of this experiment, then, application of growth substances will not substitute for other pretreatment such as light, low temperature, or carbon dioxide in bringing about germination of lettuce seed at an unfavorable temperature.

Tree peony seeds also represent a special type of germination response in that, in a given seed sample, the roots appear over quite a long period of time in spite of the fact that they are given a germination temperature which appears optimum. No method has been found, up to the present time, by which the seeds can all be induced to germinate at approximately the same time.

Tree peony seeds were, therefore, treated with solutions of potassium  $\beta$ -indoleacetate and potassium  $\alpha$ -naphthaleneacetate as noted for tomato and lettuce seed above and placed in moist granulated peat moss at a daily alternating temperature of 15° to 30° C. for germination. In no case was germination either hastened or increased. Potassium  $\alpha$ -naphthaleneacetate 360.0 mg./l. produced a harmful effect. Other treatments were ineffective. The dry seeds used as controls germinated at the same time and to the same extent as the water-soaked control so no soaking effect was evident.

#### EFFECT ON THE FURTHER GROWTH OF THE PLANT

*General results.* In several instances after the germination tests were complete in the experiments described above, measurements of seedlings were made in the very young stages while in other cases seedlings were transplanted and grown in soil for different lengths of time and the effects of seed treatment noted.

For fresh tomato seeds in two different series and for six-year-old tomato seeds stored dried at room temperature, measurements of seedlings

were made after they were treated and placed on moist filter paper at 20° C. for germination. In all cases the entire length of the seedling was taken, and ten plants from each treatment were measured. The totals of the measurements for ten plants of each treatment of fresh tomato seeds in one series are shown in Table III. Seedlings were measured four and six days after they were placed in the germinator. Very little effect on seedling length was noticed after the potassium  $\beta$ -indoleacetate treatments, except a slight decrease as a result of soaking in a concentration of 320.0 mg./l. For the potassium  $\alpha$ -naphthaleneacetate-treated seeds, however, the depressing effect of the higher concentrations on growth was evident when the seedlings were measured four days after being placed on filter paper for germination. This effect still persisted after six days.

TABLE III

LENGTH OF SEEDLINGS PRODUCED ON MOIST FILTER PAPER FROM SEEDS PRESOAKED FOR 24 HOURS IN VARIOUS SOLUTIONS. EACH FIGURE REPRESENTS, IN MILLIMETERS, A TOTAL OF 10 PLANTS MEASURED

Treatment		Tomato		Egyptian wheat			
Solution	Concn., mg./l.	Entire seedling length		Root		Shoot	
		4 days	6 days	4 days	6 days	4 days	6 days
Potassium $\beta$ -indoleacetate	320.0	215	992	208	357	65	230
	106.6	309	1083	238	443	76	287
	35.5	388	1146	310	527	84	307
	11.8	270	1011	304	486	79	288
	3.7	383	1199	291	503	63	279
Potassium $\alpha$ -naphthalene- acetate	320.0	33	106	83	170	64	263
	106.6	62	186	82	213	61	199
	35.5	74	369	107	259	74	247
	11.8	174	683	154	315	70	252
	3.7	223	977	205	347	79	295
	1.2	299	1038	251	424	71	257
Water	—	378	1188	281	480	84	300
None	—	261	1017	116	316	26	201

In the second series of tomato seed tests, measurements of the seedlings were made after seven and eleven days in the germinator. Here seedlings from both fresh and old viable seeds were used. Again solutions of potassium  $\beta$ -indoleacetate had no appreciable effect and the depressing effect on the growth of plants from seeds treated with the stronger solutions of potassium  $\alpha$ -naphthaleneacetate was noted. After eleven days, however, there was some indication that the initial retarding effect on seedling growth caused by treatment of seeds with 3.7 mg./l. of potassium  $\alpha$ -naphthaleneacetate was disappearing and the seedlings more nearly approached those of the controls in size.



Measurements similar to those for tomato were made for both Egyptian and Marquis wheat. In these cases root and blade length were measured separately. In Series 1, measurements were made four and six days after treatment for Egyptian wheat. It will be noted (Table III) that the potassium  $\beta$ -indoleacetate-treated seeds produced plants which compared favorably in length with the control lots, but the stronger concentrations of potassium  $\alpha$ -naphthaleneacetate inhibited growth of both root and shoot.

In Series 2, Marquis wheat was used and measurements made after seven and eleven days in the germinator. The same trends as for Egyptian wheat were evident. It should be pointed out that Egyptian wheat seed was less sensitive than Marquis wheat seed to injury by the substance as shown by germination percentages.

The seeds, germinated on filter paper, as described in Experiment 4 above, produced seedlings some of which were measured for root length three, four, and six days after placing in the germinator. It will be recalled that potassium  $\alpha$ -naphthaleneacetate solutions in concentrations of 320.0, 106.6, 35.5, 11.8, 3.7, and 1.2 mg./l., as well as Rootone and Merck dust 5 mg./g. were used in these tests. Dry and water-soaked seeds served as controls.

There were many cases in which some inhibition of seedling growth resulted from treatment of the seeds with growth substances. In only one case, that of the French marigold, were no effects of any kind noted. In two cases, radish and garden pea, there seemed to be an advantage to the soaking process since seedlings from dry seeds were not as large as from those which were soaked in water. Zinnia and garden pea seeds were most sensitive to growth substance treatments. All such treatments of the former and all except one of the latter resulted in injury.

The growth of eggplant, radish, and stock seedlings after certain seed treatments seemed to have been stimulated as shown by the measurements of the seedlings on filter paper three, four, and six days after treatment. None of these effects was evident in the soil plantings, however, while the injurious effects were shown plainly in the soil as well as in the filter paper plantings and in some cases persisted to the maturity of the plant.

Figure 1 which shows the germination of certain tomato seeds also shows that there was some stimulation of growth rate in seedlings from seeds treated with potassium  $\alpha$ -naphthaleneacetate, 3.7 mg./l., when a comparison is made with the water control. Auxan- and Rootone-treated seeds also appear larger than the corresponding dry controls at this time.

Ten plants from each of the treatments were transplanted to larger pots and allowed to continue growth. The apparent initial advantage in growth manifested by treatment with potassium  $\alpha$ -naphthaleneacetate,

3.7 mg./l., as well as Auxan- and Rootone-treated seeds disappeared after a period of two months. The harmful effect of the treatment with strong solutions of potassium  $\alpha$ -naphthaleneacetate was, however, still evident at this stage in the stunted growth of the seedlings.

Since all of these tomato seeds had been planted in a rather poor soil composed of sod soil, sand, and granulated peat moss in equal parts, it was thought advantageous to repeat the experiment reported above using the same type of soil and in addition a comparable lot of seeds planted in good



FIGURE 1. The effect of tomato seed treatment on germination and further growth of the plants. A. Potassium  $\beta$ -indoleacetate. B. Potassium  $\alpha$ -naphthaleneacetate. Seeds planted (1) dry; after soaking for 24 hours in (2) water, (3) 320.0, (4) 106.6, (5) 35.5, (6) 11.8, and (7) 3.7 mg./l. of solutions; dusted with (8) Auxan, (9) Rootone.

garden sod. Solutions of potassium  $\alpha$ -naphthaleneacetate alone were used in this test. Since, in the preceding experiment potassium  $\alpha$ -naphthaleneacetate, 3.7 mg./l., seemed to give an initial stimulatory effect in growth, a weaker solution, 1.2 mg./l., was added in this experiment.

There were no significant differences in germination percentages in the two types of soil. This, however, was to be expected. The real differences were to be looked for in the further growth of the plants. These seeds were planted on October 21, 1938, and the resulting seedlings photographed November 22, 1938. The plants are shown in Figure 2. The difference in growth of seedlings in the two soil types is evident when A is compared with B. In addition to the increased height in good soil, these seedlings were a darker

green and had in general a more healthy appearance. It should also be noted that the dry seeds planted in this series did not lag behind the water control in growth but actually exceeded the latter.

Some of these seedlings were also transplanted and allowed to grow to maturity. Again, as in the previous case, any possible stimulatory effects in the early stages of growth disappeared as the seedlings increased in size. In this series replicate lots of five pots each for both water and dry controls were planted in order to get some qualitative measurement of the variations



FIGURE 2. Comparative effects of tomato seed treatments when plantings were made in (A) poor soil, and (B) good soil. Seeds planted (1) dry; after soaking for 24 hours in (2) water, (3) 320.0, (4) 106.6, (5) 35.5, (6) 11.8, (7) 3.7, and (8) 1.2 mg./l. of potassium  $\alpha$ -naphthaleneacetate.

in growth within any one group. These replicates are shown in Figure 3. A glance at this figure shows one how very difficult it is to attach any significance to small qualitative differences in growth. All of the pots in each of these sets received exactly the same treatment except that the individual positions on the greenhouse bench varied. They were placed in different positions among the entire lot of treated pots. All of the replicates agreed closely in germination percentages but varied in the growth rates.

All of the flats of grain plants from Experiment 5 were grown further but no differences in growth rate or time of maturity were noted for meadow fescue, perennial rye grass, Kentucky Blue grass, oats, spring rye, or Marquis wheat. Soybean seedlings from seeds treated with Merck



powder preparations, 5 and 8 mg./g., appeared more vigorous than any of the other treatments. No quantitative measurements were made on any of these plants.

Effects of seed treatment on further growth and flowering of certain plants have also been studied.



FIGURE 3. Variations in growth of five replicate pots of (A) dry seeds, and (B) seeds soaked in water for 24 hours before planting.

Seedlings from French marigold, larkspur, nasturtium, and zinnia seeds treated and planted February 28, 1939, the germination results of which are shown in Table I, were grown in the greenhouse to the flowering stage.

The number of French marigold plants grown to maturity from each treatment varied from 40 to 55. The number of flowers produced from April 19 to May 3 were recorded and the results are shown in Table IV, where the figures represent the average number of flowers per plant. No differences in flower production were obtained. However, there were qualitative



differences in the appearance of the seedlings of the several lots, which are perhaps worthy of note. Stems of seedlings from all seeds treated with solutions of growth substances were smaller in diameter and had less red color than those of the remainder of the lots, including water and dry and dust treatments, which had heavy strong stems with many large branches, making the plants much more stiff. The liquid-treated seedlings also tended to have fewer branches and the flowers extended above the foliage whereas in all the other lots there was much branching of the plants and most of the flowers were borne below the highest foliage.

TABLE IV

EFFECT OF GROWTH SUBSTANCE TREATMENT OF SEEDS, BY SOAKING 24 HOURS IN SOLUTIONS OR BY DUSTING, ON FLOWER FORMATION. FIGURES REPRESENT THE AVERAGE TOTAL NUMBER OF FLOWERS PER PLANT

Plants		Soaked in solutions (mg./l.) potas- sium $\alpha$ -naphthaleneacetate							Dust treatments			
Grown	Name	320.0	106.6	35.5	11.8	3.7	1.2	Water control	Merck 5 mg./g.	Root- tone	Talc	None
In green- house	Larkspur	1.5	1.7	2.3	2.0	1.8	2.0	2.1	1.6	1.9	—	1.7
	French mari- gold	1.1	0.8	1.2	1.3	1.1	1.4	1.8	1.5	1.6	—	1.4
	Nasturtium	8.1	8.4	8.1	6.0	7.0	8.7	3.9	8.4	6.4	—	5.8
	Zinnia	2.8	3.3	2.2	2.4	3.1	3.1	2.4	1.9	1.8	—	2.7
In out- side plots	Lemon Ball marigold	25.6	19.3	55.4	41.7	39.6	39.4	45.5	35.2	41.8	37.9	48.6
	Orange Ball marigold	34.6	36.4	46.4	36.2	35.1	32.3	36.1	31.8	47.0	37.6	52.2
	Snapdragon	20.3	16.2	18.6	16.4	17.1	19.1	17.2	17.4	18.0	—	19.9
	Stock	29.4	31.4	42.1	36.7	31.2	27.1	29.3	33.0	30.6	—	36.7
	Zinnia	18.5	15.2	13.8	12.5	11.4	10.0	11.0	11.6	14.1	10.3	9.9

On May 3, 1939, all of these marigold plants were badly infested with thrips and it was of interest to note that plants from Merck 5 mg./g.- and Rootone-treated seeds seemed to be only slightly injured by the thrips while all of the rest were badly injured. Plants from seeds treated with 320.0, 106.6, and 35.5 mg./l. potassium  $\alpha$ -naphthaleneacetate were slightly less injured than the others. No further study has been made of this and such study would be necessary to determine whether this effect was due to chance or whether there is some correlation between treatment of seeds and seedling resistance to insect injury. This was not noted in any other case since these plants were the only ones to become thrip-infested.

Similar counts of flowers produced were made for ten plants each of larkspur, nasturtium, and zinnia, the results of which are shown in Table IV. No foliage differences were noted in these cases.

Seedlings of snapdragon, stock, Lemon Ball and Orange Ball marigold, and zinnia from the seed treatments shown in Table I were transplanted to

beds outside for flower data for the summer of 1939. Twenty-five seedlings of snapdragon from each treatment, except for 320.0 mg./l. where only 9 seedlings were available, 20 seedlings each of stock, 20 each of Lemon Ball, 25 of Orange Ball, and 35 zinnia were used. Records of the number of flowers produced were taken throughout the flowering season. Results are shown in Table IV. No significant differences were found in the number of flowers produced per plant. Furthermore, seed treatment had no effect on the percentage of plants producing flowers in any one group. Even in cases where the dose of growth substance was strong enough to reduce germination and dwarf the young plant, those seedlings that survived behaved in a manner similar to all the others as regards flowering.

Accuracy in the interpretation of such data is complicated by factors, such as seed variation, seedling selection, position in bed, etc., difficult to measure and evaluate, but the present data indicate that under the conditions of this experiment no effect on flowering was shown.

Conflicting reports on the efficacy of seed treatments with growth substance to increase growth and yield of plants have appeared in the literature from time to time. Čajlachjan and Ždanova (3) studied the effect of soaking seeds of summer wheat, winter wheat, oats, millet, summer vetch, hemp, white mustard, perilla, flax, and peas in 10, 25, and 50 mg./100 cc. solutions of  $\beta$ -indole acetic acid on further stages of development, that is, budding, earing, flowering, and ripening, and found plants from seeds treated with heteroauxin lagged behind as compared with controls. This they attributed to retardation of germination. In no case did they get accelerated ripening and concluded that growth hormones play no decisive part in plants passing from the vegetative growth to reproductive development.

Davies, Atkins, and Hudson (5) also found subsequent growth of oats, mustard, and cress plants retarded by seed treatment with  $\beta$ -indolyl propionic or  $\beta$ -indolyl acetic acid. A second test (6) using more indole derivatives gave the same general results.

Shibuya (18) treated seeds of cotton and peanut with  $\beta$  indole-acetic acid solution (20 mg./100 cc. water) and noted the after-effects of the treatment on plant growth. He found cotton plants from treated seeds flowered earlier and possessed an increased number of flowers. In the case of the peanut the vegetative period was not shortened, and, in some instances, was prolonged but in either case the number of flowers was increased.

Stier and du Buy (20) found that they could obtain an acceleration of the time of anthesis of the flowers and greater yield of fruit from tomato plants with a combination of seed treatment with auxin-talc dust mixtures and subsequent treatment of the plants with solutions of either indolyl-butyric or naphthylacetic acid at the time of field transplanting.

James (13) found a favorable effect of Rootone treatment of snapdragon, Bismarck stock, and certain amaryllid seeds on vegetative growth of the seedlings, but seeds of Iridaceae showed no noticeable reaction.

Thimann and Lane (21) soaked seeds of oats, wheat, and tomato in water for 48 hours first and then in auxin for 3, 24, and 48 hours and obtained increased growth and hastening of flowering in resulting plants. They believe that there is a relation between auxin after-effects and vernalization.

Tovartnitskij and Rivkind (22) "hormonized" seeds of spring wheat, vetch, and sugar beet with 75, 50, 25, and 10 per cent urine, with and without yarovization. Wheat and beet were favorably affected, while vetch was entirely unresponsive. The authors think this work proved that "hormonization" could be used practically for increasing the yield of agricultural crops.

Józefowicz (14) studied the effect of different seed treatments on the yield of tomatoes and found that a water bath of 45° to 46.5° C. for two and three hours increased and hastened yield; a water bath of 6° for 17 hours produced an earlier-maturing crop; and 24-hour preswelling in water of 15° to 17° increased yield. This work emphasizes the need for carefully planned controls for soaking tests.

Grace (10) claimed that the usual solution method of applying phytohormones retarded growth but that if a small, gradual supply of the active material is made available, there is a resulting increase in both root and top growth. It was claimed that this gradual supply could be obtained by using an adsorbent dust carrier for the substance. Using this method he obtained increased growth of wheat, barley, and soybean roots 14 days after planting. In a 1938 report (11), Grace presented similar effects.

Youden (23) treated wheat and soybean seeds with growth substances in talc preparations and found no stimulating effect on germination, seedling height, root systems, wet weight of tops, or yield of grain.

The results obtained here from the several experiments with different kinds of seeds agree with those of Čajlachjan and Ždanova (3), Davies, Atkins, and Hudson (5, 6), and Youden (23), in that no significant stimulating effects on vegetative growth, maturity, or flower production were shown. On the other hand, retarding effects as a result of treatment of seeds with the higher concentrations of growth substances were easily demonstrable.

*Special effects of radish seed treatment.* The response of radish seed to treatment by growth substances was first studied in a general survey of the response of different seeds to such treatment and the germination results are included in Table I. As far as the germination results were concerned there was no unusual response of these seeds to treatment. However, when the radish seedlings produced in the greenhouse were allowed to grow for



six weeks and then harvested, it was noted that some of the seedlings from seeds treated with the stronger concentrations of growth substances possessed tap roots with a peculiar double effect. A region of constriction had appeared after the root started to enlarge and below this constriction a second enlargement occurred (Fig. 4). A split, from which numerous small fibrous roots grew, was observed on many of these double roots.

For this experiment, Crimson Giant Globe radish seeds were used. To study further this doubling effect a supply of French Breakfast radish seeds were obtained from W. Atlee Burpee Seed Company on April 14, 1939. Since it had previously been found that seed size (Galloway 8, Gleisberg 9, Rotunno 17) affected the resulting seedlings, the entire seed lot was graded for size. The large size, hereafter called radish #1, would not pass through a mesh sieve with openings of 2.38 mm. or 0.0937 inch. One hundred seeds of this lot weighed 1.1104 g. The medium-sized seeds, called radish #2, passed through the sieve described above but would not pass through a mesh with openings of 2.00 mm. or 0.0787 inch, while radish #3, or the smallest seeds, passed through the 2.00 mm. sieve. One hundred seeds of radish #2 weighed 0.7969 g., while 100 seeds of radish #3, weighed 0.5473 g.

Depth of planting is another factor which has been reported to affect tap-root formation of radishes (Edmond 7). Consequently, in this experiment the seeds were all covered to a measured depth of one-half inch. Plantings were made in light, sandy soil rich in organic matter, in small flats, 10×13 inches on the inside, and three rows of 25 seeds each were spaced evenly in each flat. Duplicate flats were used for each test.

The seeds were soaked for 24 hours at 20° C. and planted with solutions drained off but without washing on April 20, 1939. They were kept in a greenhouse with an average temperature of approximately 21° C. In addition to the concentrations used in the previous test, others (200, 150, 100, 75, 50, and 25 mg./l.) were added in order to note the effect on tap-root formation of intermediate concentrations. One lot of seeds of each size group which had been soaked in water for 24 hours was exposed to vapors of naphthaleneacetic acid under a bell jar for 40 minutes before planting. In addition to the soaking treatments, dust treatments including talc, Rootone (1938), Rootone (1939), and Merck dust 5 mg./g. were used. Water-soaked seeds were used as controls for the liquid series and both dry and talc-dusted seeds were used as controls for the dust-treated series.

The germination results are indicated in part A of Tables V and VI where the actual number of seedlings from each lot of 75 seeds is given. Duplicate lots are given separately so that some idea of the error involved may be obtained. No significant increase in germination percentage over that of the controls was found. However, the decrease in germination after treatment with 320.0 mg./l., and possibly with 200 mg./l., was significant.



TABLE V  
EFFECT OF SOAKING RADISH SEED IN SOLUTIONS OF POTASSIUM  
 $\alpha$ -NAPHTHALENEACETATE (MG./L.)

Seed size	320.0	200.0	150.0	106.6	100.0	75.0	50.0	35.5	25.0	Water	Average
A. Number of seedlings produced in the greenhouse from duplicates of 75 seeds each											
#1	49	52	64	62	60	63	70	65	58	65	60.8
	50	50	57	62	63	66	69	62	63	64	60.6
#2	49	51	61	63	60	66	67	68	70	66	62.1
	47	56	54*	66	58	66	69	64	71	62	61.3
#3	51	57	60	68	63	59	69	52	66	65	61.0
	48	48**	64	63	64	68	66	62	68	67	61.8
Average	49.0	52.3	60.0	64.0	61.3	64.7	68.3	62.2	66.0	64.8	

B. Total cotyledon diameters, in millimeters, of 15 seedlings, in duplicate, for each treatment

#1	124	139	145	151	160	162	182	177	188	211	164
	131	123	162	148	165	167	177	176	207	214	167
#2	112	123	122	131	157	154	163	155	173	191	148
	105	102	118	136	131	148	164	149	170	189	141
#3	91	93	104	121	123	122	139	149	155	161	126
	92	103	96	107	133	128	118	143	141	159	122
Average	109	114	125	132	145	147	157	158	172	188	

C. Total first leaf diameters, in millimeters, of 15 seedlings, in duplicate, for each treatment

#1	134	185	184	329	424	376	464	409	501	534	354
	90	242	239	249	276	341	423	403	507	487	326
#2	83	158	153	263	311	363	436	386	515	507	318
	121	82	99	333	305	297	438	409	457	495	304
#3	90	188	120	243	235	300	340	414	441	506	288
	82	150	115	173	265	264	323	356	395	477	260
Average	100	168	152	265	303	324	404	396	469	501	

\* 66 seeds used.

\*\* 62 seeds used.

No differences were found in the germination capacity of the various sizes of seeds.

The diameters of the cotyledons at the widest point on each was taken on April 25, 1939, five days after planting. Seedlings were chosen at regular intervals so that 15 seedlings were measured in each duplicate flat, or 30 seedlings were measured for each treatment. These measurements are shown in part B of Tables V and VI. It is apparent that seed size affected cotyledon size. This, of course, was to be expected. The largest cotyledons were found in seedlings produced from the largest seeds and the smallest

TABLE VI  
EFFECT OF TREATING RADISH SEEDS WITH DUSTS CONTAINING GROWTH SUBSTANCES

Seed size	Rootone (1938)	Rootone (1939)	Merck 5 mg./g.	Talc	Dry	Average
A. Number of seedlings produced in the greenhouse from duplicates of 75 seeds each						
#1	63	60	59	61	58	60.2
	64	65	63	58	65	63.0
#2	58	65	66	62	63	62.8
	63	64	60	62	58	61.4
#3	63	62	70	64	68	65.4
	66	67	68	64	69	66.8
Average	62.8	63.8	64.3	61.8	63.5	
B. Total cotyledon diameters, in millimeters, of 15 seedlings, in duplicate, for each treatment						
#1	210	208	194	198	244	211
	207	178	184	188	237	199
#2	196	176	186	187	204	190
	168	181	174	177	194	179
#3	172	165	147	164	165	163
	156	156	145	162	165	157
Average	185	177	172	179	202	
C. Total first leaf diameters, in millimeters, of 15 seedlings, in duplicate, for each treatment						
#1	493	601	500	555	589	548
	479	594	511	593	515	520
#2	536	544	468	495	533	515
	522	482	513	542	524	517
#3	459	540	479	522	570	514
	467	458	452	508	513	480
Average	493	522	487	536	541	

cotyledons in seedlings produced from the smallest seeds. This applied to both moist and dry series.

An additional effect on cotyledon size was also found as a result of liquid treatment (Table V B). The strongest solution used resulted in smallest cotyledons regardless of seed size and there was a gradation in increasing cotyledon size with decreasing concentration. In no case, however, did the cotyledons of any of the treated seeds exceed in size those of the controls. Seeds which had been soaked in water for 24 hours and then exposed to naphthaleneacetic acid vapors produced seedlings with cotyledon size approximating those from seeds soaked in solutions of 100 mg./l. or 75 mg./l.

On the other hand, no effect on cotyledon size resulted from treatment

of seeds with dusts containing the growth substances, when compared with the talc control although differences in cotyledon size with seed size can again be demonstrated in this experiment (Table VI B). All six of the dust-treated seed lots show slight decrease in cotyledon size as compared with the six dry controls.

On May 9, 1939, the diameter of the first leaf at its widest point was taken (part C of Tables V and VI). The same general results were obtained as for cotyledon size but by this time the depressing growth effect of the stronger concentrations was even more evident (Table V C). Again, in no case was a stimulating effect found. Seed size continued to exert an influence easily demonstrable at this time.

On April 27, or seven days after planting, seedlings were removed from each flat leaving only 10 seedlings evenly spaced in each row, or 30 seedlings per flat. These were allowed to continue growing until May 26, 1939, at which time they were harvested and the wet weights of both tops and roots recorded for each row (except one) of 10 plants. This means that three rows of one flat and two rows of its duplicate or a total of 50 plants were weighed from each treatment. These data have been summarized in Table VII.

TABLE VII

EFFECT OF RADISH SEED SOAKED 24 HOURS IN SOLUTIONS OF POTASSIUM  $\alpha$ -NAPHTHALENE-ACETATE (EXPRESSED AS MG./L.) ON THE WET WEIGHTS OF TOPS AND ROOTS OF SEEDLINGS. FIGURES REPRESENT TOTALS OF 5 LOTS OF 10 PLANTS EACH FROM EACH TREATMENT

	Seed size	320.0	200.0	150.0	106.6	100.0	75.0	50.0	35.5	25.0	Water	Average
Top weights (grams)	#1	156	201	260	237	249	266	303	306	255	327	256
	#2	132	253	206	253	254	258	242	300	273	322	249
	#3	112	231	152	201	236	245	251	218	225	281	215
Average		134	229	206	230	247	256	265	275	251	310	
Root weights (grams)	#1	171	203	205	204	331	294	397	420	378	489	318
	#2	134	200	160	326	304	248	353	396	433	444	300
	#3	91	221	121	222	253	272	297	353	325	441	260
Average		132	208	162	281	296	271	339	390	379	458	

Using top and root weights, then, as criteria for evaluating possible differences we again find that there is an indication of seed size effect, the largest seeds giving the highest wet weights of both tops and roots, but the water control produced heaviest tops and roots as compared with treated lots.

Doubling occurred over quite a range of treatment concentrations as is shown in Figure 4, in which this phenomenon is shown after treatment of the seeds with concentrations of 200, 100, and 50 mg./l. of potassium  $\alpha$ -naphthaleneacetate. The harvest of one entire row is pictured in each case.



FIGURE 4. Radish roots produced 36 days after planting from seeds soaked for 24 hours in solutions of potassium  $\alpha$ -naphthaleneacetate. A. 200.0, B. 100.0, C. 50.0, and D. 25.0 mg./l., or E. water.



Some doubling effects were also noted for concentrations as low as 35.5 mg./l., but occurred only occasionally with solutions as weak as 25 mg./l. Naphthaleneacetic acid vapor treatment of water-soaked seeds also produced double roots. In no case was a similar effect produced in either dry or water controls. Only a few double roots were produced by dust treatments and these instances were not capable of reproduction under the conditions of our experiment whereas liquid treatments of the effective concentrations invariably produced doubling. Individual seed plantings varied from 10 to 70 per cent of doubling, the larger number always resulting from treatments in the 50 to 200 mg./l. range.

In spite of the lack of any quantitative data indicating any sort of a stimulatory effect of growth substances applied as seed treatment, some apparently stimulative qualitative effects were noted for radish treated with concentrations of potassium  $\alpha$ -naphthaleneacetate of 25 mg./l. or less. This is shown in Figure 4, if one compares the size of the tap roots from 25 mg./l. treatment with those from water treatment. A similar effect for a solution of only 1.1 mg./l. was shown in another series. Seeds dusted with dust 5 mg./g. (made from Merck 35 mg./g. by diluting with talc) and with Rootone (1938) also produced tap roots of increased size as compared with dry controls. These differences were noted for three separate tests of radish. Failure to obtain increased weights in these larger roots was due to their pithy texture.

Amlong and Naundorf (1) reported that four-year-old radish seed treated with water and 0.01 N heteroauxin were planted in the garden, where the treated seeds gave stronger and larger plants. They also reported bigger roots and tops from seeds treated with  $10^{-4}$  N and  $10^{-3}$  N but no increase with  $10^{-2}$  N. Similar results were obtained with sugar beets. No doubling effect of tap root was noted by these workers.

The results with radishes raised the question of whether other plants forming tap roots would respond in a like manner. Seeds of beets, carrots, and turnip were therefore treated with 320.0, 106.6, 35.5, 3.7, 1.2, 0.4, and 0.13 mg./l. solutions with no stimulatory effects on germination, and further growth of the seedlings indicated no effect on tap-root formation. These seeds were also germinated after having been dusted with Merck dusts of 2 mg./g., 5 mg./g., 8 mg./g., and 12 mg./g. concentrations of indolebutyric acid with corresponding talc and dry controls with no effect either on germination or subsequent growth.

#### SUMMARY

Seeds of *Allium cepa*, *Antirrhinum majus*, *Avena* sp., *Beta vulgaris*, *Brassica rapa*, *Daucus carota* var. *sativa*, *Delphinium ajacis*, *Festuca pratensis*, *Glycine max*, *Lactuca sativa*, *Lolium perenne*, *Lycopersicon esculentum*, *Matthiola incana*, *Paeonia suffruticosa*, *Phytolacca decandra*, *Pisum*

*sativum*, *Poa pratensis*, *Raphanus sativus*, *Secale cereale*, *Solanum melongena* var. *esculentum*, *Tagetes erecta*, *T. patula*, *Triticum* sp., *Tropaeolum majus*, and *Zinnia* sp. were treated with growth substances applied as vapors, liquids, or dusts, and the effect on germination and further growth of the plants noted.

Soaking in solutions of potassium  $\alpha$ -naphthaleneacetate at concentrations of 320.0, 106.6, 35.5, 11.8, 3.7., and 1.2 mg./l. failed to increase the speed or percentage of germination. Injury was obtained with the higher concentrations. Powder treatments with Auxan, Rootone, or Merck preparations (containing indolebutyric acid in concentrations of 2, 5, 8, or 12 mg./g.) were also without a stimulating effect. Similar treatments of six-year-old tomato seeds failed to reveal any advantage in germination.

Measurements of very young seedlings grown from treated seeds of nine different varieties indicated increased seedling length as a result of certain treatments of eggplant, radish, and stock. These differences were apparent only when the seeds were germinated on moist filter paper and never appeared in soil plantings. Stunting effects were obtained by one or more of the higher concentration treatments of all nine types of seeds.

When tomato plants from treated seeds were grown to maturity, no stimulation of growth was noted, although some stunting effects persisted. Treated and control lots of seeds of meadow fescue, perennial rye grass, Kentucky Blue grass, oats, spring rye, and wheat produced plants with the same growth rate and time of maturity.

A study of the flowering of larkspur, marigold, nasturtium, snapdragon, stock, and zinnia plants from treated seeds revealed no significant differences from the control plants, either in the number of plants producing flowers or in the number of flowers produced.

In many of the tap roots of radish plants from seeds treated with the higher concentrations a region of constriction was produced after the root started to enlarge and below this constriction a second enlargement occurred giving the appearance of a double root.

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## SEED TREATMENTS WITH TALC AND ROOT-INDUCING SUBSTANCES

W. J. YODEN

### INTRODUCTION

Certain substances, notably indolebutyric acid, indoleacetic acid, and naphthaleneacetic acid, have been found useful in stimulating the formation of roots on cuttings (4). It is not surprising that attempts would be made to apply these same chemical compounds to seeds in the expectation that there would be some measurable response in the seedling or mature plant. Reports have been published (2, 3, 5, 6) which give instances of large increases in the root system and to a lesser extent increased yields. The ease with which these materials may be applied to seeds, especially in the form of powders, coupled with the low cost per unit treated, makes the idea an extremely attractive possibility. Furthermore, it is a current practice to dust many seeds with various mercury preparations, and the further addition of a small quantity of these root-inducing materials to the powder would add nothing to the work of treating the seeds.

At the very outset of an investigation to determine whether or not there is any merit to the proposal to treat seeds with these preparations, it is apparent that there is the widest latitude in the matter of procedure. Presumably seeds of economic interest would be selected. The powders found useful for treating cuttings afford a natural choice, but it is evident that the concentration of active substance in the inert carrier may be varied within wide limits. Either the amount of powder per unit of seed or the proportion of active substance in the carrier or both may be varied. There is the possibility that soaking the seeds in solutions of the substance may be effective. Finally, having treated the seeds in a variety of ways, it is necessary to execute either greenhouse or field trials to ascertain the result. At the beginning, when it is desirable to test numerous treatments, pot tests in the greenhouse are more economical than field plots which would involve a large expenditure of effort and which in some respects are not as convenient for following the early development of the plant. In any event it will be of interest to record observations of the germination, shoot height, root system, weight of plant or yield, and any unusual characters which may develop.

This paper gives an account of a number of experimental trials which, while they by no means encompass all the possibilities, do survey with exactitude a considerable range of conditions. The work has been confined to wheat seeds (*Triticum aestivale* L. var. Marquis Beardless) and soybean seeds (*Glycine max* Merr. var. Early Wilson Black) and to treatment of

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these seeds in the dry state with powders. It has further been limited to three known organic substances using talc as the carrier and one commercial preparation available on the market. Both untreated seeds and seeds dusted with talc were planted as controls. The amount of active material in the talc was varied from 0.025 to 3.2 per cent and the proportion of powder to seeds from 0.2 to 2.0 per cent. The amount of active substance included extremes as low as 0.5 and as high as 240 parts per million of the weight of the seeds. Most of the plants were grown in the greenhouse using both good and poor soils and sand with nutrient solution. In all cases germination and wet weight of tops of the plants were obtained. Also the heights of thousands of wheat seedlings were measured in some of the experiments. The root systems were examined when grown in sand culture. Field trials were conducted with both wheat and soybean and in the case of the wheat, the stand and air-dry weight of both straw and grain recorded. The stand and wet weight of tops of the soybeans were also obtained.

It must be admitted that even a moderate advantage, if found, would be of consequential importance when applied on the large scale to which these treatments lend themselves. Every effort was therefore made by replication and the utilization of modern experimental designs for yield trials to reduce the error of the comparisons to a minimum. Indeed, it became one of the purposes of the work to accumulate evidence showing that quantitative measurements on plants grown in the greenhouse may be greatly improved by the orientation of the test pots in a manner similar to that now accepted as essential in field trials. Inasmuch as some of these arrangements are somewhat complicated, the detailed discussion of them has been incorporated in a parallel paper (7). This permits the more concise presentation of much of the evidence since only the final averages are given.

It cannot be claimed that these treatments were without any effect. It will be apparent, however, upon examination of the series of experiments listed below that the desired effects, if any, were too small to manifest themselves even in experiments particularly directed to disclose the accuracy of greenhouse trials. And more especially in those cases where a convenient series of concentrations permitted the calculation of a trend curve, there were no indications that would give any encouragement to the contention that the plants grown from seeds treated in the dry state with the common root-inducing substances excelled those grown from untreated seeds. Barton (1) has similarly failed to find any advantage from the use of root-inducing substances on seeds.

#### GREENHOUSE AND FIELD EXPERIMENTS

*Experiment 1, greenhouse.* Seven treatments were applied to Early Wilson Black soybeans and the seeds in lots of 50 planted in gallon jars. The seven treatments were untreated control, talc, Rootone (a commercial

powder used for treating seeds and cuttings), and indolebutyric acid at the rates of 20, 40, 80, and 160 parts per million of the weight of seeds. The dry seeds were tumbled for ten minutes in bottles containing the powders. Talc preparations of the indole compound were made up containing 4, 8, 16, and 32 mg. per gram of mixture, and these powders used at the rate of 0.5 per cent of the weight of seeds. The Rootone and talc were also applied in amounts of 0.5 per cent. Two similar sequences of treatments were arranged at rates of application of 1 and 2 per cent of the powders. Indole preparations containing 2, 4, 8, 16, and 1, 2, 4, 8 mg. per gram were used so that the final amount of this substance was identical in all three series. Finally, each of the three series was planted in three soils. A somewhat deficient soil (A) was used for one set of 21 pots, the same soil plus manure (B) for a second group of 21 pots, and this last soil with an addition of phosphate (C) for the last 21 pots.

The seeds were planted May 3 and five days later 2547 seedlings had developed from the 3150 seeds. The germination was remarkably consistent,  $\chi^2$  for the 63 lots of 50 seeds was found to be 61.7, a value which would be exceeded 50 per cent of the time.

The total seedlings obtained from the three soils were 842, 855, and 850, and from the three rates of powder application, 839, 841, and 867. The total number of seedlings from the seven different treatments are given in Table I. The computed value of  $\chi^2$  for these counts is 8.28.

TABLE I  
SOYBEANS GROWN IN SOIL IN GREENHOUSE

Observation	Treatment						
	Control	Talc	IB* 20	IB 40	IB 80	IB 160	Rootone
Experiment 1, seedlings	369	378	361	373	355	351	360
Height, mm. May 12	45	46	46	47	48	50	44
Soil A Wt. g. May 23	43	40	43	45	41	45	44
Soil B Wt. g. May 23	71	70	67	72	68	66	76
Soil C Wt. g. May 23	70	69	65	73	69	63	68
Soil A Wt. g. June 3	55	55	59	63	54	50	68
Soil B Wt. g. June 3	160	145	148	157	146	172	160
Soil C Wt. g. June 3	173	136	179	157	174	172	172
Experiment 2, seedlings	106	98	109	105	106	103	98
Total wt. g.	188	169	189	191	184	170	169

\* Indolebutyric acid, p.p.m.

It was noted that seedlings from seeds treated with the highest concentration, i.e., 160 parts per million, of indolebutyric acid showed hyponasty of the cotyledon leaves.

The pots were thinned to 15 plants and on May 12 the height to the cotyledon of every one of the 945 plants was measured. The average of the

135 seedlings for each treatment is shown in Table I. The tops of ten plants were cut from each pot on May 23 and the total wet weight of the 30 plants from each treatment for each soil is given in Table I. The uniformity of these results is in decided contrast to the total weights when classified by soils which were 301, 490, and 477 grams. The five plants remaining in each pot were cut and weighed June 3, and while the weights of 15 plants are not as uniform as the preceding weights, they do not show any gain from the treatments. The same plants grouped by soils in order of fertility gave weights of 403, 1088, and 1162 grams. The total weight of the plants from seeds treated with 0.5 per cent of powder was 874 grams. This is in close agreement with the weights of 889 and 890 grams found for the parallel series where the powders were used at the rates of 1 per cent and 2 per cent respectively. These three series have been combined to obtain the values given in Table I.

*Experiment 2, greenhouse.* One week after the soybean seeds used in the above experiment had been treated with the powders, a second planting was made of all the 21 combinations using a good soil. Duplicate pots of 20 seeds were planted. Since the three series of 0.5, 1, and 2 per cent of powder application were indistinguishable, the net result was 6 replicates of each of the seven treatments. These plants were not thinned, but on May 26, 16 days after planting, the tops from all 42 pots were weighed and counted. The last two lines of Table I show the values obtained. The standard error of the weight totals is 6.4 grams and the differences obviously without significance.

*Experiment 3, field.* Talc preparations of naphthaleneacetic acid and indolebutyric acid at the rate of 4 mg./g., Rootone, talc, and control were compared at two different rates of application to the seeds. The powders were used in the quantities 150 and 300 mg. per 20 g. of seeds, corresponding to 30 and 60 parts per million, and both these replicated with the further addition of 100 mg. of Semesan, a powder containing 30 per cent hydroxymercurichlorophenol. The seeds were planted immediately after they had been rotated for ten minutes in bottles containing the powder.

The seeds were planted May 25, four inches apart, in rows 30 inches apart. The unit row was 8 feet long. The two rates of application both with and without the mercury disinfectant formed a plot of four rows for each treatment. The five treatments were arranged in a Latin Square with one column missing. The condensed results of the harvest on August 11 are shown in Table II which gives the stand and total yield in tops for the several combinations. The only contrast which gave significant odds was that between the mercury-treated seeds and the seeds without disinfectant. Naphthaleneacetic acid gave the lowest yield, but was only 5 per cent below the average for all treatments.

*Experiment 4, field.* Eleven different lots of wheat seeds were prepared



according to Table III which also shows the code letters assigned to the treatments and the yields obtained per plot.

TABLE II  
STAND AND TOTAL FRESH WEIGHT IN OUNCES OF TOPS OF SOYBEAN PLANTS

Application mg./20 g. seed		Composition of powder				
Powder	Semesan	Control	Talc	Indole- butyric acid 4 mg./g.	Naphtha- leneacetic acid 4 mg./g.	Rootone
150	0	83*-654	69-698	69-618	71-581	68-594
300	0	80-690	71-600	67-595	67-563	75-635
150	100	84-651	72-626	78-688	75-658	83-723
300	100	68-600	76-643	77-650	73-638	79-702
Average		79-649	72-642	73-638	72-610	76-664

\* The first entry is the stand obtained from 100 seeds.

TABLE III  
COMPOSITION OF POWDERS AND AVERAGE YIELDS FOR EXPERIMENT 4. YIELD  
FIGURES FROM TABLE IV

Code letter	Material	Mg./g. of talc	Powder g./100 g. seed	P.p.m.	Seme- san g./100 g. seed	Corrected plot yield, g.	
						Grain	Straw
A	Indolebutyric acid	5	1	50	—	146	650
B	Semesan	—	—	—	0.5	138	620
C	Indolebutyric acid + Semesan	2	1	20	0.5	134	626
D	Talc	—	1	—	—	135	627
E	Indolebutyric acid + Semesan	5	1	50	0.5	130	639
F	Indolebutyric acid	12	1	120	—	133	624
G	Rootone	?	1	—	—	132	616
H	Talc + Semesan	—	1	—	0.5	127	579
I	Indolebutyric acid + Semesan	12	1	120	0.5	133	629
J	Control	—	—	—	—	140	621
K	Indolebutyric acid	2	1	20	—	139	649

The seeds were planted May 3 in 66 plots each four by three feet. Each plot contained six rows, four feet long, six inches apart. Fifty seeds were counted into each row. The seedling counts (May 18) were made on the four center rows, and these four rows were harvested on July 22. These observations are recorded in Table IV which further shows the arrangement of the plots in incomplete blocks of six treatments which provide for six replicate plots of each treatment. The analysis of variance showed that a very large part of the variation was due to soil heterogeneity and was accounted for by the incomplete blocks. Both for the grain and straw the mean square for treatments was found to be slightly less than the error mean square. The corrected average plot yields in grams for grain and straw are shown in Table III.

TABLE IV

FIELD ARRANGEMENT OF WHEAT PLOTS SHOWING STAND AND AIR-DRY YIELDS OF STRAW AND GRAIN IN GRAMS. SEE ALSO EXPERIMENT 4 AND TABLE III

B 139 795 118	I 181 735 141	D 168 720 111	F 148 725 132	C 161 745 168	G 137 645 157	K 170 690 168	E 154 610 147	A 179 445 123	J 151 390 105	H 164 395 103
E 137 810 129	A 171 735 157	G 144 755 134	I 174 795 162	F 144 695 142	J 142 640 139	C 142 650 146	H 154 605 150	D 162 360 96	B 158 325 90	K 169 440 122
G 135 795 145	C 151 615 127	I 158 790 128	K 147 735 107	H 162 600 108	A 180 710 164	E 159 625 151	J 156 685 170	F 174 410 111	D 183 400 112	B 159 410 115
H 151 745 161	D 158 745 153	J 150 710 152	A 141 760 143	I 171 705 163	B 168 615 144	F 161 635 157	K 174 700 165	G 127 370 93	E 155 405 112	C 157 475 133
D 133 830 146	K 136 805 157	F 131 790 129	H 154 790 144	E 155 770 159	I 151 600 138	B 164 700 165	G 114 575 126	C 149 315 83	A 179 360 98	J 130 420 108
I 159 805 111	E 154 760 114	K 136 740 152	B 181 810 189	J 135 695 159	C 166 755 149	G 155 690 158	A 177 670 170	H 154 315 89	F 177 365 111	D 157 395 114

*Experiment 5, greenhouse.* Five of the eleven treatments from the preceding trial were used for this experiment. Fifty seeds were planted in good soil in gallon jars and five jars planted for each lot of seeds. The 25 jars were arranged in a Latin Square according to the arrangement given in Table V. This table shows the code letter of the treatment, the average heights of the seedlings 12 days after planting, the wet weight of the tops three weeks later, and the number of plants surviving. The average heights in millimeters, the average weight of tops in tenths of a gram, and the total number of plants were as follows:

	A	B	D	G	J
Average height	165	173	159	160	162
Average weight	380	404	379	379	346
Total number	201	215	200	199	186

It is of interest that G, which had the poorest stand in the field (812 as against an average of 934), gave no indications in the greenhouse of germinating below the others. The control treatment (J) in the field gave a count of 864 and was second low. Detailed examination of these figures failed to reveal any differences among the treatments. Both the row and

TABLE V

ARRANGEMENT OF TEST JARS IN GREENHOUSE AND OBSERVATIONS ON THE AVERAGE HEIGHT IN MM., WEIGHT OF TOPS IN DECIGRAMS, AND NUMBER OF PLANTS

J	D	B	G	A
156	163	171	166	147
337	376	368	369	349
41	43	45	38	38
D	G	A	B	J
158	161	178	167	159
354	433	427	462	422
40	38	45	45	39
G	B	J	A	D
161	179	169	174	151
326	494	248	370	332
42	42	34	40	38
A	J	G	D	B
166	175	166	163	176
362	374	364	417	302
39	37	40	39	45
B	A	D	J	G
170	159	162	150	145
393	394	416	350	402
38	39	40	35	41

column totals of the Latin Square in the case of the weights showed considerably greater variation than the treatment totals.

*Experiment 6, greenhouse.* Thirteen lots of wheat seeds were tumbled with a series of powders to obtain treatments at the rate of 0.5, 2, 4.5, and 8 parts per million for each of the three chemicals, indoleacetic acid, naphthaleneacetic acid, and indolebutyric acid. The appropriate powders were applied in the amount of 40 mg. to 20 g. of seeds. One lot was tumbled with talc, and served for three plantings to provide a control lot for each series. Seven short rows across each of 15 flats were each planted with 20 seeds. Seven out of the 15 treatments occurred in every flat and were so selected and ordered within the flats as to constitute a Youden Square (7). There was no difference in stand and these counts as well as the details of the weights and arrangement will be omitted. The corrected total weight in grams of the tops, 26 days after planting, for each of the 15 treatments is presented in Table VI. The overall average weights for the three chemicals are almost identical, but there is a slight suggestion (supported by the analysis of variance with small odds) of a concentration effect.

*Experiment 7, greenhouse.* The indication in Experiment 6 of a concentration effect of the treatments led to another trial of the same chemicals at concentrations of 1, 2, 3, and 4 parts per million. Two lots were treated with talc, and two others, a control untreated, and a Rootone-treated lot were added. The powders were applied at the rate of 40 mg. per 20 g. of

TABLE VI

CORRECTED WET WEIGHTS IN GRAMS OF THE TOPS OF WHEAT SEEDLINGS OBTAINED FROM 140 SEEDS (EXP. NO. 6) AND FROM 80 SEEDS (EXP. NO. 8)

Experiment No.	Chemical in talc	Concentration, p.p.m.				
		0	0.5	2	4.5	8
6	Indoleacetic acid	73	72	54	64	58
	Naphthaleneacetic acid	61	68	63	66	61
	Indolebutyric acid	75	67	65	57	59
8	Indoleacetic acid	68	76	66	52	
	Naphthaleneacetic acid	72	88	76	88	
	Indolebutyric acid	74	70	77	60	
	No treatment	85				

seeds. The seeds were planted in six-inch pots, 20 seeds to a pot, in sand culture, and were replicated six times. The 96 pots were arranged in a Youden Square and observations taken on the germination, heights, and weights. Omitting all details the corrected weights taken one month after planting are listed in Table VII. Again, although the ratio of mean square for treatments to the error mean square is only 1.26, there is a hint of slightly lower yields with the two highest concentrations of the chemicals. Nevertheless, the results can only be interpreted as a remarkably uniform series of observations, none of which differs by as much as 10 per cent from the general average.

TABLE VII

CORRECTED WET WEIGHTS IN GRAMS OF THE TOPS OF WHEAT SEEDLINGS GROWN FROM 120 SEEDS

Chemical in talc	Concentration, p.p.m.				
	0	1	2	3	4
Indoleacetic acid	210	201	205	194	206
Naphthaleneacetic acid	—	199	203	208	208
Indolebutyric acid	205	204	183	192	199
No treatment	211				
Rootone			185		

*Experiment 8, greenhouse.* This experiment was a repetition of Experiment 6. Only part of the seeds treated at the time were used in Experiment 6. The surplus seeds were kept for two months in the same bottles in which they had been tumbled. These seeds were then planted after they had been in contact with the powder for two months. The seeds were planted in six-inch pots, 20 in a pot. The pots were replicated four times and germination, heights, and weights obtained. The weights are entered in the lower part of



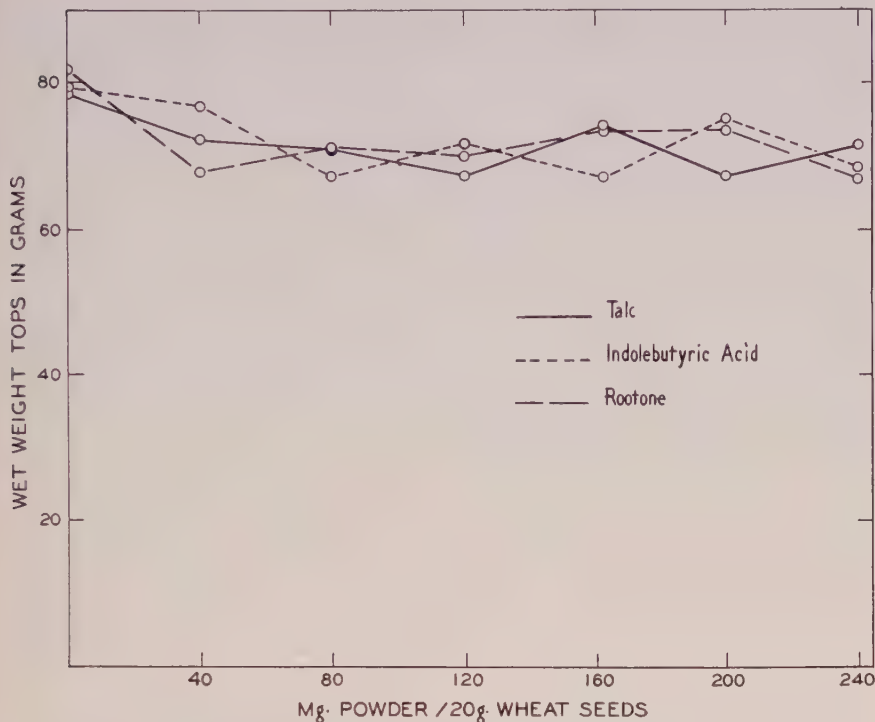


FIGURE 1. Total wet weight after 23 days of tops obtained from 100 wheat seeds treated with increasing amounts of talc, indolebutyric acid in talc, and Rootone.

Table VI and yield no indication that prolonged contact of powder and seed prior to planting is a factor to be considered.

*Experiment 9, greenhouse.* Three powders, talc (325 mesh), Rootone, and talc containing 20 mg. of indolebutyric acid per gram of powder, were used to treat wheat seeds. Eighteen 20-gram lots of seeds were rotated for 20 minutes with 40, 80, 120, 160, 200, and 240 mg. of each of these three powders. (These values are also the p.p.m. of indolebutyric acid.) A con-

TABLE VIII

CORRECTED WET WEIGHTS, IN DECIGRAMS, OF WHEAT SEEDLINGS GROWN FROM 100 SEEDS

Powder	Milligrams of powder per 20 g. seeds							Average
	0	40	80	120	160	200	240	
Talc	783	722	710	675	742	675	717	718
Rootone	818	679	712	700	733	735	671	721
Indolebutyric acid	793	767	672	718	672	751	688	723
Average	742	723	695	698	716	720	692	721



FIGURE 2. Root systems from 105 pots, each planted with 20 wheat seeds. The five rows are replicates. From left to right the first seven treatments were 0, 40, 80, 120, 160, 200, and 240 mg. per 20 g. of seeds of talc containing 2 mg. per gram. The next seven treatments were made with the same series of quantities of Rootone powder, and the last seven treatments with an identical series for talc.

trol lot of seeds without powder was similarly rotated to provide three lots, one for each series. Samples of 50 seeds were planted in 105 pots of sand, there being five replicates of each treatment. The pots were arranged on the greenhouse bench in a Youden Square, and a record made of the seedling count, height, and weight. These measurements can be found in a paper (7, p. 223, Table I) discussing the accuracy of greenhouse trials. The total wet weights of the plant tops in decigrams are tabulated in Table VIII. The analysis of variance of these results shows, as would be expected, no difference between the three powders or any differential response at the several concentrations. The odds are slightly better than 20 to 1 for a concentration effect. The curves in Figure 1 give a consistent impression. The roots from the entire experiment were removed and photographed. Figure 2 shows there is no evidence of any substantial effect upon the root system.

*Experiment 10, greenhouse.* A 20-g. sample of wheat seeds was rotated with 120 mg. of talc for ten minutes and these planted in 28 pots of sand, 20 seeds to the pot. A similar set of pots containing control seeds was planted and paired off on the greenhouse bench. The tops when weighed showed a total excess of weight of control over talc treatment of 5.6 grams. The average difference per pot was 0.20 gram with a standard error of 0.27.

#### DISCUSSION

The conclusions drawn from the individual experiments are in general agreement as to the absence of beneficial effect of the substances tested. The results do not preclude the possibility that with other methods of application it might be possible to demonstrate an effect. Grace (2) has claimed remarkable effects with these same materials when used in powder form at concentrations within the range reported in this investigation. The experiments given above, while limited to two varieties, have been performed under a number of different conditions as regards the growing medium and the environment. The most intensive efforts were directed towards a study of the concentration of substance applied, without detecting any band at which the material might profitably be used. The only positive evidence points to the superiority of the untreated control seeds. The several experiments have been reviewed to contrast the talc-coated seeds with the control seeds. There are 19 comparisons available, and in all but three of these the control lots gave weights in excess of those from the talc-treated seeds. On the average the talc-treated lots were about 5 per cent below the control lots and a difference of this size would not be established with any certainty in single experiments. The similarity of the curves in Figure 1 suggest that the inert carrier is responsible for the indicated trend.

## SUMMARY

A study was made to determine whether root-inducing substances influence seed germination and seedling growth.

Wheat and soybean seeds were treated in the dry state with indoleacetic acid, naphthaleneacetic acid, and indolebutyric acid, talc, and Rootone (commercial preparation) and grown in sand and soil in the greenhouse and in the field. The concentration of the organic compounds in the talc preparations, as well as the proportion of powder to seeds, was adjusted to cover the range 0.5 to 240 parts per million of active substance by weight of the seeds. In a series of ten experiments observations were made of the germination, seedling height, wet weight of tops, yield of grain, and root systems, and no significant case was found in which the germination and growth of the treated lots exceeded the controls. On the contrary the aggregate of evidence points to slightly lower values for the treated lots, and indicates that these are the result of the presence of the talc. Nineteen contrasts of talc-treated seeds with controls gave fifteen cases in which the controls were superior and one tie. On the average, the excess weight of the control plants was about 5 per cent.

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# EXPERIMENTAL DESIGNS TO INCREASE ACCURACY OF GREENHOUSE STUDIES

W. J. YODEN

## INTRODUCTION

For many years agricultural field experiments which were conducted to compare different varieties or fertilizers or other variations in cultural practice often gave unsatisfactory and non-reproducible results. This in large measure was due to the heterogeneity of the soil. Some of the methods resorted to in an effort to eliminate this difficulty still persist—such as the removal and thorough mixing of a quantity of soil which is then returned to containers sunk in the ground. Needless to say such measures were both expensive and inadequate, as the very nature of the procedure departed from ordinary methods of growing crops. Both these limitations were more appropriately met by the adoption of suitable arrangements of the test plots. The simplest of these is the replicated block design. In this case one each of all the items in the experiment is assigned at random to equivalent regions or plots which together form a compact block. Each complete replication constitutes a block and it is a requirement that the size of the block be kept within such limits as will insure a reasonable uniformity of conditions within the block. On the other hand, considerable differences between the blocks are not troublesome and that portion of the total variance due to such differences may be easily segregated from the estimate of error. The Latin Square arrangement is another device and is often extremely efficient because it permits the formation of twice as many blocks as replications. The fact that the blocks, as the size of the square increases, are no longer compact but long strips, limits the number of items which may be compared by this arrangement.

It is undesirable to reduce the unit plot size below a certain point as it is necessary to provide for a sufficient number of plants to give a representative sample of the plants. Further, practical requirements of planting and harvesting cannot be ignored in the choice of the plot dimensions.

The development of a technique for coping with non-uniform material or environment is illustrated by certain ingenious arrangements which evade the restriction in the number of treatments compared, while keeping the block small enough to maintain its uniformity. Yates in two papers (5, 6) has developed arrangements which permit the comparison of considerable numbers of treatments with blocks containing incomplete replications and hence made up of small numbers of plots. In one very useful type the condition governing the selection of treatments which are to com-

prise the several blocks is that every possible pair of treatments must be found in some one or other of the blocks. Consider the  $7 \times 7$  Latin Square in which all seven treatments are found in each of the seven rows and the seven columns. There are seven replications of the treatments.

A	B	C	D	E	F	G
B	C	D	E	F	G	A
D	E	F	G	A	B	C
<hr/>						
C	D	E	F	G	A	B
E	F	G	A	B	C	D
F	G	A	B	C	D	E
G	A	B	C	D	E	F

Note that this Latin Square can be divided into two portions, one of three rows and one of four rows. The upper portion now consists of seven abbreviated columns, each of three letters, and these may be taken as incomplete blocks. Each block of three letters affords three pairs such as AB, AD, BD, and the seven blocks a total of 21 pairs which are all the different pairs which can be formed from the seven letters. Yates (5) showed that for such incomplete blocks a simple arithmetical procedure partitions the total variance into that due to treatments, to differences between blocks, and a residual variance or error. The above arrangement goes one step further and shows that complete replications of all the treatments can constitute horizontal rows. Thus there may also be eliminated from the total variance that portion arising from differences among these rows.

The remainder of the original Latin Square in turn provides for incomplete blocks of four treatments and each of the possible 21 pairs appears twice. Either of these arrangements has the advantage of reducing the number of replications. In an actual field trial the incomplete blocks would preferably be arranged in more compact form such as:

C	E	A	D	E	G	A	B	C	D	C	D	D	F
F	G	G	F	A	B	C	F	B	G	E	A	E	B

and this would likely outweigh the relinquishment of the complete replications. In certain situations it has proved to be of considerable advantage to preserve them. In studies involving individual leaves on plants (7), each plant constituted an incomplete block, and each leaf level (such as the top leaf of each plant) a complete replication. In this case there was such a marked gradient within the plant that a large portion of the variability of

the leaves arose from their position on the plant and it was important to take this into consideration.

#### DESIGN OF GREENHOUSE EXPERIMENTS

In some recent greenhouse studies of the effect of dusting seeds with various chemicals incorporated in talc it was found that the customary greenhouse bench set up conditions which made it desirable to utilize both the incomplete blocks and the complete replications. A greenhouse bench is usually built against the side of the house and is narrow in comparison with its length. The width of the bench accommodates a limited number of pots, perhaps not more than six, so that the pots are inevitably arranged in long rows. If each of these rows constitutes a replication then differences in, say, light and heat received by the row nearest the glass and the row nearest the aisle are fairly constant and set up a natural grouping by rows. The incomplete blocks in turn deal with any gradient in conditions which may be found between the ends of the bench since the several pots in any short row across the bench receive the same environment in respect to such a trend. These gradients were found on benches in the center of a greenhouse. Some examples will now be given to demonstrate the efficiency of these arrangements in greenhouse studies.

*Example 1.* In this experiment wheat (*Triticum aestivale* L. var. Marquis Beardless) seeds were treated with 16 different dusts. Six pots, each with 20 seeds, were planted from each treatment. The arrangement on the greenhouse bench was as follows, where the letters indicate the several treatments:

B	I	G	N	L	A	O	M	E	K	P	C	H	J	D	F	400.2
G	O	N	M	C	B	I	F	L	D	E	A	P	K	H	J	522.4
H	K	B	E	J	C	D	I	N	P	G	M	L	F	A	O	561.1
I	E	P	J	O	D	F	N	A	C	H	G	B	L	K	M	574.5
J	M	O	G	H	E	L	C	P	I	F	K	D	B	N	A	605.9
A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	548.7
																3212.8

The values at the right show the total weight in grams of the green tops from each complete replication. The extent to which these rows differ is illustrated by the value of over 600 for the fifth row as against 400 for the first row. The following analysis of variance of the plant weights obtained in this experiment shows that the complete replications account for nearly as much of the total variation as the incomplete blocks, and these together, at the expense of 20 degrees of freedom out of the 95, account for 84 per cent of the total variation; the dusts being without effect.

## Analysis of Variance

	D. F.	S. S.	M. S.
Complete replications	5	1611.5	322.30
Incomplete blocks	15	1996.9	133.13
Dusts	15	164.0	10.93
Residue	60	521.4	8.69
Total	95	4293.8	

The efficiency of these balanced incomplete blocks, which is a measure of the loss of information if there is no actual basis for the formation of such blocks, is  $E = \frac{1 - 1/K}{1 - 1/V}$ , where  $K$  is the number of pots per block, and

$V$  the number of dusts under test. In this case  $E = 8/9$ , and the loss inherent in the design is unimportant in comparison with the reduction in the residual mean square to 8.7 from the value 33.5 which would have been found if the sum of squares for incomplete blocks were included in the residue.

*Example 2.* Another experiment with wheat seeds treated with 13 dusts, there being four replications, gave the following result for the fresh weights:

## Analysis of Variance

	D. F.	S. S.	M. S.
Complete replications	3	132.17	44.06
Incomplete blocks	12	379.02	31.59
Dusts	12	246.49	20.54
Residue	24	315.68	13.15
Total	51	1073.36	

Here again the complete replications have proved at least as valuable as the blocks in reducing the error mean square.

*Example 3.* In this case wheat seeds were treated with three different chemical dusts. The dusts were applied at the rates of 0, 40, 80, 120, 160, 200, and 240 mg./20 g. of seeds, and the 21 combinations replicated five times. Table I shows the actual arrangement of the pots and in each case the number of plants obtained from 20 seeds, the average height and the height of the median plant seven days after planting, and the total fresh weight in grams of the plant tops 17 days later. The analysis of variance for the heights and weights shows that the complete replications removed a greater fraction of the total variance in the case of the weights. This is not surprising as the heights were taken three or four days after the seedlings first became visible and before the location had any considerable effect. In this, as in all the examples, the per cent of the seeds germinating



TABLE I

MEASUREMENTS MADE ON WHEAT SEEDLINGS GROWN FROM SEEDS TREATED WITH CHEMICALS INCORPORATED IN TALC. THE 105 POTS, EACH PLANTED WITH 20 SEEDS, WERE ARRANGED IN GREENHOUSE IN THE ORDER SHOWN IN THE TABLE

Replication 1				Replication 2				Replication 3				Replication 4				Replication 5			
P*	AH	MH	N	W	P	AH	MH	N	W	P	AH	MH	N	W	P	AH	MH	N	W
R 1	105	122	16	78	H 3	112	110	18	118	H 4	114	115	20	111	T 1	105	114	16	110
H 3	98	110	19	98	R 3	100	125	17	120	T 2	108	127	16	127	T 4	111	121	16	125
H 4	103	110	17	107	T 5	113	121	18	138	R 3	110	130	18	133	R 5	115	128	19	137
T 1	118	125	10	130	R 2	130	120	20	153	R 4	119	140	19	159	H 0	118	126	18	148
T 0	119	127	17	134	R 6	125	129	17	146	T 3	130	141	18	144	T 6	117	121	18	155
R 3	113	127	16	131	R 1	93	116	16	106	T 0	125	129	20	104	H 1	116	125	19	168
R 0	128	130	18	130	R 4	124	132	18	168	H 5	112	115	18	133	R 6	112	118	19	156
H 6	136	133	17	140	H 4	104	129	17	114	R 6	119	131	16	142	T 2	120	131	15	147
H 1	110	123	10	149	T 1	121	133	18	167	T 4	117	124	18	173	H 2	106	120	18	153
T 4	108	122	18	137	T 0	113	120	18	149	H 6	92	120	15	132	R 4	113	119	20	151
T 5	92	112	16	103	T 3	116	122	16	133	R 1	117	121	19	168	R 2	107	120	17	157
T 3	117	123	16	127	H 6	144	143	15	152	H 2	114	131	19	169	H 0	106	126	19	160
R 2	112	119	17	129	H 3	101	115	16	141	H 1	117	123	17	174	H 3	95	120	17	161
T 2	114	124	16	143	H 2	106	105	18	160	T 6	108	117	16	144	R 1	94	120	16	145
R 4	93	119	17	128	H 1	84	98	15	129	H 5	100	117	17	159	H 4	104	105	18	149
H 2	79	99	16	101	H 5	102	111	18	156	R 2	105	118	16	133	T 0	120	129	20	205
T 6	103	110	18	136	T 4	94	109	16	132	H 0	114	128	18	177	R 0	108	115	19	179
H 5	89	108	16	131	T 6	105	108	16	156	T 5	97	119	16	154	H 6	91	109	16	137
R 6	91	105	15	106	H 0	116	127	19	178	R 5	120	119	18	150	H 5	101	100	18	168
H 0	111	108	20	158	T 2	101	109	15	140	T 0	112	112	20	166	T 5	99	113	17	159
R 5	107	109	20	134	R 0	100	113	18	130	T 1	93	101	17	116	T 3	93	105	15	137
2255 2471 363 2635				2313 2513 359 2986				2361 2578 371 3134				2272 2493 372 3213				2196 2422 364 3164			

\* P = Powder; R = Rootone; H = Hormodin; T = Talc alone; Numerals 0, 1, 2, 3, 4, 5, 6 indicate respectively 0, 40, 80, 120, 160, 200, 240 milligrams of powder per 20 grams of seeds.

AH = Average height of seedlings in mm. 7 days after planting.

MH = Median height same measurements.

N = Number of plants weighed.

W = Fresh weight of tops in decigrams 23 days after planting.

was not affected by the treatments, since the observed frequencies are in accord with samples drawn from a homogeneous population.

#### Analysis of Variance

	D. F.	Av. Heights		Med. Heights		Weights	
		S. S.	M. S.	S. S.	M. S.	S. S.	M. S.
Complete replications	4	733.0	183.3	624.8	156.2	104.84	26.21
Incomplete blocks	20	5651.7	282.6	5411.3	270.6	185.25	9.26
Chemicals	2	155.6	77.8	302.1	151.1	0.22	0.11
Amounts of dust	6	842.5	140.4	428.1	71.4	39.68	6.61
Chem. $\times$ Amts.	10	1144.3	114.4	449.2	44.9	21.64	2.16
Dummy treatments	2	43.8	21.9	34.7	17.4	1.13	0.57
Residue	60	5491.2	91.5	2418.7	40.3	147.89	2.46
Total	104	14062.1		9668.9		500.65	

*Example 4.* Fifteen lots of wheat seeds were dusted with three chemicals each at five concentrations. Incomplete blocks of seven were formed by planting seven short rows across a wooden flat. Fifteen flats were arranged side by side in a row so that any set of 15 rows in a line, say those at the north end of each flat, constituted a complete replication. In spite of the flats constituting natural blocks, the position of the row within the flat was still worth taking into consideration as is shown by the following analysis of the weights of the plant tops.

#### Analysis of Variance

	D. F.	S. S.	M. S.
Complete replications	6	51.50	8.58
Incomplete blocks	14	41.16	2.94
Chemicals	2	2.08	1.04
Concentrations	4	34.20	8.55
Chemicals $\times$ Conc.	6	15.89	2.65
Dummy treatments	2	15.55	7.78
Residue	70	182.68	2.61
Total	104	343.06	

The two last examples, which are of factorial design, show the chemicals are indistinguishable in their action. There is evidence (odds better than 50 to 1) of a slight effect due to concentration. In the case of Example 4 the combined weights for the three chemicals are as follows:

Concentration in p.p.m.	0	0.5	2.0	4.5	8.0
Wet weight of tops in g.	208.4	206.5	182.3	187.6	178.1

ARRANGEMENTS OF BALANCED INCOMPLETE BLOCKS

Tables XVIII and XIX of Fisher and Yates' Statistical Tables for Biological, Agricultural and Medical Research (3) list a number of balanced incomplete block arrangements. There remains the further orientation of the units within the blocks to bring about complete replications running across the blocks. This is possible where the number of blocks is equal to the number of items to be tested and the number of replications also are equal to the number of units in a block. The following patterns, sometimes known as Youden Squares (2, 4, 1, 6) show the required orientation for a number of useful cases besides those given above. The columns (and rows) of that portion of the square selected should be permuted to insure the removal of any systematic pattern in these squares.

11 treatments, 5 or 6 replications

A	G	I	K	J	H	B	F	C	E	D
B	A	H	I	K	G	F	C	D	J	E
C	F	A	G	E	B	D	K	J	I	H
D	J	F	A	H	C	K	E	I	B	G
E	C	B	D	A	K	J	I	H	G	F
-----										
F	E	D	C	B	A	I	H	G	K	J
G	H	E	J	C	F	A	B	K	D	I
H	D	G	B	I	J	C	A	E	F	K
I	K	C	F	G	D	E	J	A	H	B
J	B	K	E	D	I	H	G	F	A	C
K	I	J	H	F	E	G	D	B	C	A

13 treatments, 4 or 9 replications

A	B	C	D	E	F	G	H	I	J	K	L	M
C	D	E	F	G	H	I	J	K	L	M	A	B
D	E	F	G	H	I	J	K	L	M	A	B	C
H	I	J	K	L	M	A	B	C	D	E	F	G
-----												
B	C	D	E	F	G	H	I	J	K	L	M	A
E	F	G	H	I	J	K	L	M	A	B	C	D
F	G	H	I	J	K	L	M	A	B	C	D	E
G	H	I	J	K	L	M	A	B	C	D	E	F
I	J	K	L	M	A	B	C	D	E	F	G	H
J	K	L	M	A	B	C	D	E	F	G	H	I
K	L	M	A	B	C	D	E	F	G	H	I	J
L	M	A	B	C	D	E	F	G	H	I	J	K
M	A	B	C	D	E	F	G	H	I	J	K	L

## 15 treatments, 7 or 8 replications

M	E	O	L	D	K	A	B	H	J	I	N	G	C	F
H	N	L	K	E	I	B	C	F	D	M	G	O	A	J
L	J	K	F	H	G	C	A	D	E	N	M	I	B	O
F	G	E	I	A	B	D	M	O	K	J	C	L	H	N
G	L	H	B	N	M	E	O	J	A	F	D	C	I	K
A	B	C	D	I	O	F	N	M	L	E	H	J	K	G
I	H	F	N	O	E	G	L	B	M	C	K	D	J	A

K	D	I	O	G	F	L	J	E	H	A	B	M	N	C
D	A	B	C	M	J	I	K	N	O	G	F	H	E	L
B	C	N	A	J	L	M	I	G	F	K	O	E	D	H
E	O	D	J	L	A	N	G	C	B	H	I	K	F	M
J	M	G	H	K	D	O	F	A	C	B	E	N	L	I
C	K	A	M	B	N	J	H	I	G	D	L	F	O	E
N	F	J	G	C	H	K	E	L	I	O	A	B	M	D
O	I	M	E	F	C	H	D	K	N	L	J	A	G	B

## 16 treatments, 6 or 10 replications

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
B	G	A	H	L	J	N	P	O	K	D	C	F	M	I	E
C	H	M	A	N	O	B	L	K	F	P	J	I	E	D	G
D	I	G	K	A	M	P	B	E	L	C	O	N	J	F	H
E	J	K	N	P	A	O	D	M	B	I	H	C	G	L	F
F	A	L	O	I	P	C	M	B	N	J	E	H	D	G	K

H	C	I	B	F	D	A	E	P	G	L	N	J	K	M	O
G	D	B	F	H	E	J	A	C	M	N	K	O	L	P	I
I	E	D	C	B	N	K	O	A	P	G	M	L	F	H	J
J	M	E	G	C	H	D	F	N	A	O	P	B	I	K	L
K	P	F	E	D	C	M	N	L	O	A	I	G	H	J	B
L	K	H	J	G	I	E	C	F	D	B	A	P	O	N	M
M	L	J	I	O	G	F	K	D	C	H	B	A	P	E	N
N	F	O	P	J	B	L	I	H	E	M	G	K	A	C	D
O	N	P	L	M	K	H	G	J	I	F	D	E	B	A	C
P	O	N	M	K	L	I	J	G	H	E	F	D	C	B	A



19 treatments, 9 or 10 replications

A	N	Q	L	I	H	G	F	E	M	J	R	S	B	C	D	O	P	K
B	A	P	M	J	K	R	I	H	E	F	Q	D	S	O	C	G	N	L
C	B	A	P	L	M	K	Q	I	N	G	E	O	F	J	S	H	D	R
D	C	B	A	P	S	N	L	J	I	O	H	G	K	R	E	Q	F	M
E	D	O	B	A	Q	P	N	M	R	S	L	I	H	K	F	C	G	J
F	L	J	G	C	A	S	R	O	B	N	D	Q	I	H	P	K	M	E
G	K	F	R	Q	C	A	O	S	P	B	J	M	N	D	L	E	H	I
H	J	E	O	S	F	D	A	N	C	R	B	K	P	I	M	L	Q	G
I	M	K	H	G	R	E	D	A	Q	C	S	B	L	P	O	N	J	F

J	S	R	N	M	L	O	P	K	A	Q	F	E	D	G	H	I	C	B
K	E	S	Q	B	N	M	J	P	H	A	O	F	G	L	I	D	R	C
L	F	C	S	O	B	Q	K	R	J	H	A	N	E	M	G	P	I	D
M	G	D	K	N	J	B	S	Q	O	I	P	A	C	E	R	F	L	H
N	H	G	D	R	O	L	B	F	K	P	I	C	A	S	J	M	E	Q
O	I	H	E	D	G	F	C	B	S	L	N	J	M	A	Q	R	K	P
P	O	I	F	E	D	H	G	C	L	M	K	R	Q	B	A	J	S	N
Q	P	L	I	F	E	J	M	D	G	K	C	H	R	N	B	A	O	S
R	Q	M	J	K	I	C	H	L	D	E	G	P	O	F	N	S	B	A
S	R	N	C	H	P	I	E	G	F	D	M	L	J	Q	K	B	A	O

21 treatments, 5 replications

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U
B	F	K	M	S	A	O	C	D	E	L	H	U	P	I	R	J	Q	T	N	G
C	N	F	O	L	G	B	S	J	H	A	P	I	Q	R	M	T	K	U	E	D
D	J	U	F	Q	I	S	N	P	O	M	T	B	A	C	E	G	H	R	K	L
E	R	P	T	F	H	K	M	S	U	J	B	Q	O	L	G	C	D	A	I	N

31 treatments, 6 replications

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	a	b	c	d	e
F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	a	b	c	d	e	A	B	C	D	E
G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	a	b	c	d	e	A	B	C	D	E	F
I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	a	b	c	d	e	A	B	C	D	E	F	G	H
P	Q	R	S	T	U	V	W	X	Y	Z	a	b	c	d	e	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
T	U	V	W	X	Y	Z	a	b	c	d	e	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S

Yates and Hale (6) have pointed out that these designs may be considered as Latin Squares with two or more rows or columns missing and discuss the analysis of these designs. It is clear that the rejected rows may be utilized and this often permits some choice in the number of replications. The blocks for 15, 16, and 21 treatments are especially useful because these numbers can be factored and are hence available for a factorial system of treatments. Cornish (1) has published the formulas for the partition of the treatment sum of squares for factorial designs arranged in incomplete randomized blocks.

#### SUMMARY

It has been shown that even in the comparatively well controlled cultural conditions available in greenhouses the proper disposition of the test pots contributes markedly to the accuracy of the results obtained. In the first example it was found that 84 per cent of the total sum of squares was accounted for by differences between complete replications along the bench and differences between the incomplete blocks across the bench. Expressed another way, the standard deviation of a single pot was reduced from 7.2 to 3.1 grams. These amounts are 21.5 and 9.4 per cent respectively of 33.5 grams which is the average yield of a single pot. The examples given exemplify the effectiveness of experimental design when account is taken of the characteristics of the material or environment encountered. It is apparent that much depends upon an apt selection of an arrangement which will take the fullest possible advantage of either known or suspected similarities in the material or environment. Experimenters are usually aware of the existence of such groupings of their material but have, until recent years, been unable to make much use of these groups. This paper makes available a number of new arrangements of Latin Squares with missing rows which should be found useful in greenhouse studies.

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## SOME EFFECTS OF TREATMENT OF SEEDS WITH GROWTH SUBSTANCES ON DORMANCY

LELA V. BARTON

In view of the recent interest shown in the general effects of seed treatment with growth substances, some tests were made using seeds which normally exhibit some delay in germination.

### MATERIAL AND METHODS

Four types of seeds were used. Seeds of the flowering dogwood (*Cornus florida* L.), the red-osier dogwood (*Cornus stolonifera* Michx.), the domestic apple (*Pyrus* sp.), and the Purple crab apple (*Pyrus malus* L. var. *niedwetzkyana* Aschers. and Graebn.), all of which fail to produce seedlings in the greenhouse unless pretreated, were studied. Seeds of the osage orange (*Maclura pomifera* Raf. Schneider) and the American elm (*Ulmus americana* L.), which germinate to some extent when planted directly in the greenhouse, but which produce more prompt and complete seedling stands after certain pretreatments, were also used. Seeds of the Florida runner peanut (*Arachis hypogaea* L.), which are dormant when freshly-harvested but which lose this dormancy with a period in dry storage, also served as test material. Germinated seeds of the gold-banded lily (*Lilium auratum* Lindl.), the tree peony (*Paeonia suffruticosa* Andr.), and certain *Viburnum* sp. were treated with growth substances to determine the effect on epicotyl dormancy.

Except in the case of the peanut, all growth substance treatments were with liquid solutions of various concentrations. For the most part the seeds were soaked, and after the solutions were drained off, they were planted without washing. Certain dust treatments were used for peanut seed.

Since low temperature pretreatment is a common method used to overcome dormancy, it was used in these tests. The seeds were mixed with moist granulated peat moss and kept in a constant temperature chamber of 5° C. This method has been referred to as stratification.

Seeds were soaked in solutions of growth substances before and after different stratification periods. Their germination was then tested either immediately or after a second stratification period.

### EXPERIMENTAL RESULTS

A preliminary test was begun in April, 1937, using apple seeds. Phenylacetic acid was used in concentrations of 200 and 100 mg./l. and the seeds were soaked for a period of 44 hours at room temperature before planting. Water-soaked seeds were used as controls. Some of the seeds were planted

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in soil immediately while others were mixed with moist granulated peat moss and placed at 5° C. for after-ripening. No seedling production in the greenhouse was obtained from the planting of any seed lot without a period at 5° C., and the same seedling production was obtained from all lots after one, two, or three months at 5° C. Approximately 20 per cent was obtained after one month at 5° C. After two months, after-ripening was complete, and 80 to 95 per cent seedling production was obtained. No further advantage was gained by three months at 5° C. Similar results were obtained when dry seeds were mixed with granulated peat moss and 50 cc. of 200 or 100 mg./l. phenylacetic acid were added to the peat.

This experiment showed, then, no effect of phenylacetic acid of 200 or 100 mg./l. strength on dormant apple seeds.

In order to determine whether the length of the soaking period was important and to extend the concentration range as well as the substances used, a second experiment was performed with apple seeds of the same lot in which phenylacetic, naphthaleneacetic, indoleacetic, and indolebutyric acids were used in concentrations of 100, 50, 25, and 10 mg./l. for soaking periods of 6 hours, 48 hours, and 5 days at room temperature. In spite of a careful separation of the seeds to select the sound ones, a few split ones were included in the treatments. No doubt this fact influenced the mold which appeared within 48 hours after the soaking periods were started. The solutions were changed on the five-day-period seeds after 48 hours. At the end of five days the seeds were washed with fresh solutions, not water, before planting in peat or soil. Seeds were planted in the greenhouse immediately after such treatment and also after a period of one-half, one, and two months at 5° C. Results similar to those in the preceding experiment were obtained and are shown in Table I.

In spite of certain variations in the percentages of seedlings produced, due, at least in part, to variations in the number of split seeds in the original sample, certain definite trends were shown. One month in a moist condition at 5° C. was not sufficient to after-ripen all of the seeds. That two months were long enough for low-temperature treatment was shown by the high percentages of seedling production from plantings made in the greenhouse at that time. It was further demonstrated that soaking the seeds in concentrations of from 10 to 100 mg./l. of phenylacetic, naphthaleneacetic, indoleacetic, or indolebutyric acid before placing them in moist granulated peat moss at 5° C. did not influence their subsequent after-ripening.

Since treatment of dry dormant apple seeds with growth substances seemed to have no effect, it seemed of some interest to determine the effect of such substances on after-ripened or partially after-ripened seeds.

Potassium  $\alpha$ -naphthaleneacetate in concentrations of 200, 100, 50, 25, and 10 mg./l. was used and the soaking period was two days at room tem-



TABLE I  
EFFECT OF SOAKING DRY APPLE SEEDS IN SOLUTIONS OF GROWTH SUBSTANCES UPON  
SUBSEQUENT AFTER-RIPENING AND GERMINATION

Treatment		Per cent seedling production in greenhouse after months at 5° C.											
		0			0.5			1			2		
Solution	Concn., mg./l.	6 hrs.	48 hrs.	5 days	6 hrs.	48 hrs.	5 days	6 hrs.	48 hrs.	5 days	6 hrs.	48 hrs.	5 days
Phenylacetic acid	100	0	0	0	0	0	6	12	30	28	82	100	91
	50	0	0	0	0	0	6	8	22	20	82	87	88
	25	0	0	0	0	0	6	0	20	16	82	83	88
	10	0	0	0	0	0	0	8	16	12	92	83	81
Naphthalene- acetic acid	100	1	0	1	1	0	2	24	4	10	59	86	85
	50	0	2	0	0	0	0	8	16	16	55	84	83
	25	0	0	0	1	0	2	12	18	22	63	86	89
	10	0	0	0	0	0	0	14	30	16	57	77	87
Indoleacetic acid	100	0	0	0	0	0	2	10	26	24	71	85	90
	50	0	1	0	1	0	2	14	18	28	67	87	89
	25	0	0	0	1	0	2	14	24	18	72	90	91
	10	0	0	0	0	0	2	12	30	24	77	94	92
Indolebutyric acid	100	0	0	0	0	0	0	14	30	28	77	88	83
	50	0	0	0	1	4	0	8	18	14	79	86	98
	25	0	0	0	1	0	0	10	18	24	69	88	85
	10	0	0	0	1	8	0	12	8	28	87	85	87
Water	—	0	0	2	0	0	0	26	24	32	63	82	89

perature. In addition to apple seeds, those of *Cornus stolonifera* and *Maclura pomifera* were used. Seeds of each type were given each of the treatments after one and one-half and two and one-half months in moist peat at 5° C.

That apple seeds were fully after-ripened and ready to grow after one and one-half months in moist peat at 5° C. was shown by the fact that the water control gave 92 per cent seedling production. The germination of seeds from the same after-ripened lot was inhibited entirely by soaking in 200 or 100 mg./l. solutions and soaking in concentrations of 50, 25, and 10 mg./l. reduced the seedling production to 24, 76, and 82 per cent. Similar results were obtained with after-ripened seeds of *Cornus stolonifera* although complete inhibition of germination was not obtained in this case. After-ripened *Maclura* seeds, on the other hand, were not affected by any of the treatments. *Maclura* seeds differed from those of apple and *Cornus stolonifera* in that they were not entirely dormant, but gave approximately 50 per cent germination in the greenhouse without any pretreatment. A stratification period of one month at 5° C., however, hastened and improved seedling production.

That the soaking process was not in itself harmful was shown by a control lot of seeds transferred directly from moist peat at 5° C. to green-

house soil. The water-soaked controls compared favorably with these in seedling production percentage.

A second test similar to the first was made after the seeds had had low-temperature pretreatment of two and one-half months, a period shown to be in excess of that normally required for breaking the dormancy. Soaking of such apple seeds in solutions of 200 and 100 mg./l. allowed 2 and 20 per cent seedling production when the samples were planted in the greenhouse.

Lots of treated after-ripened seeds were then re-stratified at 5° C. to determine whether the dormancy induced by the growth substances could be again broken by a subsequent period at a favorable after-ripening temperature. They were left in 5° C. for one month after treatment. In all cases except apple soaked in 200 mg./l., where the seeds were apparently killed, this second stratification period induced germination of some of the seeds. In the case of apple seeds soaked in 100 mg./l. solution, for example, the seedling production was increased from 0 to 19 per cent by re-stratification for one month while those soaked in 50 mg./l. solution increased from 24 to 65 per cent.

The same general experiment was repeated on a much more extensive scale using the same solutions for treatment but this time using seeds of domestic apple, Purple crab apple, *Cornus florida*, and *Ulmus americana*. Seeds were soaked in growth substances before and after pretreatment in moist granulated peat moss for one, two, and three months after which they were planted directly in the greenhouse or re-stratified at 5° C. for two weeks or one, two, three, and in one case four months and then planted in the greenhouse. Results agreed with those already reported and with those to be reported below (Fig. 1).



FIGURE 1. Apple seeds after-ripened one month at 5° C., then soaked 24 hours in (1) water, or solutions of potassium  $\alpha$ -naphthaleneacetate in concentrations of (2) 200, (3) 100, (4) 50, (5) 25, and (6) 10 mg./l.

A final experiment on these stratification effects was begun in September, 1938, using seeds of the domestic apple, the Purple crab apple, and the American elm. Potassium  $\beta$ -indoleacetate and potassium  $\alpha$ -naphthaleneacetate in concentrations of 320.0, 106.6, 35.5, 11.8, and 3.7 mg./l. were used. The procedure was essentially as described above except that the soaking period was for 24 hours at 20° C. The results are shown in Table II. It is evident from a study of these data that domestic apple seeds require a longer period of after-ripening than Purple crab apple seeds but both of them require some after-ripening before seedlings will be produced in soil. A few seeds of the Purple crab apple after-ripen in two weeks at 5° C. as indicated by the production of 2 to 12 per cent seedlings upon planting in the greenhouse at this time, but at least a month is required to after-ripen seeds of the domestic apple. Both types of seeds require two months for complete after-ripening.

A certain percentage of seeds of American elm will produce seedlings in the greenhouse without any pretreatment (Table II), resembling, in this respect, those of *Maclura*. However, the stand will be improved greatly by pretreatment in moist granulated peat moss at 5° C. for two weeks or one month before planting in the greenhouse. These seeds represent a form intermediate between those requiring no pretreatment for germination and those failing to germinate without pretreatment.

Domestic apple seeds and those of the Purple crab apple were similar in their behavior toward growth substances. In Figure 2 it will be seen that no germination was obtained from domestic apple seeds treated with solutions of potassium  $\alpha$ -naphthaleneacetate before stratification. After another lot of seeds had been stratified for one month in moist granulated peat moss they were treated with potassium  $\alpha$ -naphthaleneacetate solutions, all of which reduced the subsequent seedling production in the greenhouse as compared with that of the water control. The same treatments given a lot of seeds stratified for two months, a period known to be sufficient for complete after-ripening, were less harmful except in a concentration of 320.0 mg./l.

The response of Purple crab apple seeds to the same treatments (Fig. 2) differed in that 11.8 and 3.7 mg./l. did not inhibit germination of seeds after-ripened for either one or two months. Stronger solutions, however, caused decrease in germination.

It will be seen from Table II that a period of two weeks at 5° C. in moist granulated peat moss was sufficient to result in good seedling production from greenhouse plantings of seeds of the American elm. One month pretreatment was also effective but after that germination started at the low temperature so that seed samples taken at the two-month period included some seeds which had started to germinate and were more susceptible to injury by subsequent treatment. This would account for the lower germination shown in Figure 2 from seeds stratified for two months.

TABLE II

SEEDLING PRODUCTION IN THE GREENHOUSE FOLLOWING GROWTH SUBSTANCE TREATMENT  
OF NON-AFTER-RIPENED AND AFTER-RIPENED SEEDS WITH AND WITHOUT A FURTHER  
PERIOD AT 5° C.

Seed	Months of stratification at 5° C.		Potassium $\beta$ -indoleacetate, mg./l.					Potassium $\alpha$ -naphthaleneacetate, mg./l.					Water
	Before soaking	After soaking	320.0	106.6	35.5	11.8	3.7	320.0	106.6	35.5	11.8	3.7	
Domestic apple, 25 seeds each	0	0						0	0	0	0	0	0
		0.5						0	0	0	0	0	
		1						4	20	8	16	16	12
		2						60	76	88	84	92	92
		3						76	88	—	—	—	92
	1	0						4	8	4	20	24	56
2	0.5						16	36	64	56	76	68	
	1						36	56	80	88	92	84	
3	2							8	60	—	—	—	100
	0	0						0	20	32	84	88	80
1	0.5							0	56	84	88	88	84
	1							16	56	84	—	—	80
Purple crab apple, 50 seeds each	0	0	0	0	0	0	0	0	0	0	0	0	0
		0.5	2	2	10	12	2	8	6	4	6	8	2
		1	56	46	54	50	44	10	34	42	40	50	44
		2	94	98	88	90	98	40	72	90	80	82	92
		3	—	—	—	—	—	42	80	—	—	—	88
	1	0	40	54	56	58	64	0	4	22	42	42	30
		0.5	80	88	96	88	90	10	42	74	70	70	80
		1	96	94	94	94	98	4	52	82	94	86	88
	2	2	—	—	—	—	—	10	48	—	—	—	—
		0	66	76	88	100	98	2	56	70	86	84	90
	3	0.5	88	100	96	100	100	12	50	80	98	98	94
		1	—	—	—	—	—	12	48	96	—	—	74
0	3	0	—	—	—	—	—	2	22	—	86	—	92
	0	12	12	8	14	12	17	19	15	30	32	6	
American elm, 100 seeds each	0.5	0	51	55	62	61	60	30	50	58	55	61	58
		0.5	58	77	65	71	65	26	64	61	68	48	68
		1	39	49	60	67	61	12	29	43	51	63	60
		2	—	—	—	—	—	—	—	—	—	—	—
		3	—	—	—	—	—	—	—	—	—	—	—
	1	0	68	85	86	80	81	30	65	71	87	84	65
		0.5	69	70	79	69	76	8	37	65	80	86	81
		1	54	47	67	62	48	12	21	56	56	79	70
	2	2	22	79	65	27	11	1	27	58	69	69	65
		0	54	83	88	84	78	13	20	63	93	78	89
	3	0.5	77	92	98	90	82	7	14	44	68	78	92
		1	69	—	—	—	—	7	4	27	64	—	—
0	2	0	65	83	81	88	90	5	15	45	69	80	76
	0.5	69	66	75	83	85	5	36	45	84	82	72	
1	1	—	—	—	—	—	8	12	—	—	—	—	



Some of the seeds with germination inhibited as shown in Figure 2 were replaced in moist granulated peat moss at 5° C. for longer periods to see if the dormancy induced by treatment with growth substances could be overcome. These results are shown in Table II and Figure 3. For example, domestic apple seeds treated after one month stratification at 5° C. with 320.0, 106.6, or 35.5 mg./l. potassium  $\alpha$ -naphthaleneacetate gave 4, 8, and 4 per cent seedling production respectively. These percentages could be brought up to 16, 36, and 64 by placing again at 5° C. for an additional two weeks and to 36, 56, and 80 by a month at 5° C. after growth substance treatment. The harmful effect of 3.7 mg./l. potassium  $\alpha$ -naphthaleneacetate on these seeds after they had been stratified for a month could be completely overcome by an additional two weeks at 5° C. (Fig. 3). Figure 3 shows similar effects for Purple crab apple seeds.

Elm seeds again differed in their response. In Figure 3 it will be seen that the water control gave best seedling production in the greenhouse after stratification for two weeks plus a re-stratification period of two weeks following the 24-hour soaking treatment. It was to be expected that one month re-stratification, making a total time at 5° C. of one and one-half months, would lessen the seedling production percentage for the reason noted above, namely that germination begins at 5° C. if the seeds are left longer than a month.

The germination of elm seeds, stratified for two weeks, was increased somewhat by treatment with potassium  $\alpha$ -naphthaleneacetate of 35.5, 11.8, or 3.7 mg./l. This was also noted for such treatment of non-after-ripened seeds (Fig. 2). This effect was not evident when elm seeds were treated after one or two months in moist peat at 5° C. It may be that seeds of this class, which do not exhibit complete dormancy, but which produce much better seedling stands after certain pretreatments, are capable of stimulation by certain growth substance treatments. However, much more work is needed to establish this point and the stimulation as obtained by such treatments under the conditions of this experiment was not sufficient to warrant their use as a substitute for either low-temperature pretreatment or soaking in water under a light source as a means of speeding up the germination of elm seeds.

Shibuya<sup>1</sup> used dormant peanut seed and forced their germination by applying heteroauxin (0.1 g. mixed with 1 g. lanolin) to the radicle of the excised embryo. In order to obtain germination it was necessary to scratch the base of the radicle with a small needle before applying the hormone. Wounding alone had no effect.

In view of this report, the effects of liquid and dust treatments as reported above were tried on seeds of the 1939 crop of Florida runner peanuts

<sup>1</sup> SHIBUYA, TSUNETOSHI. Forcing the germination of dormant seeds by means of growth hormone. Jour. Soc. Trop. Agric. 10: 1-8. 1938.

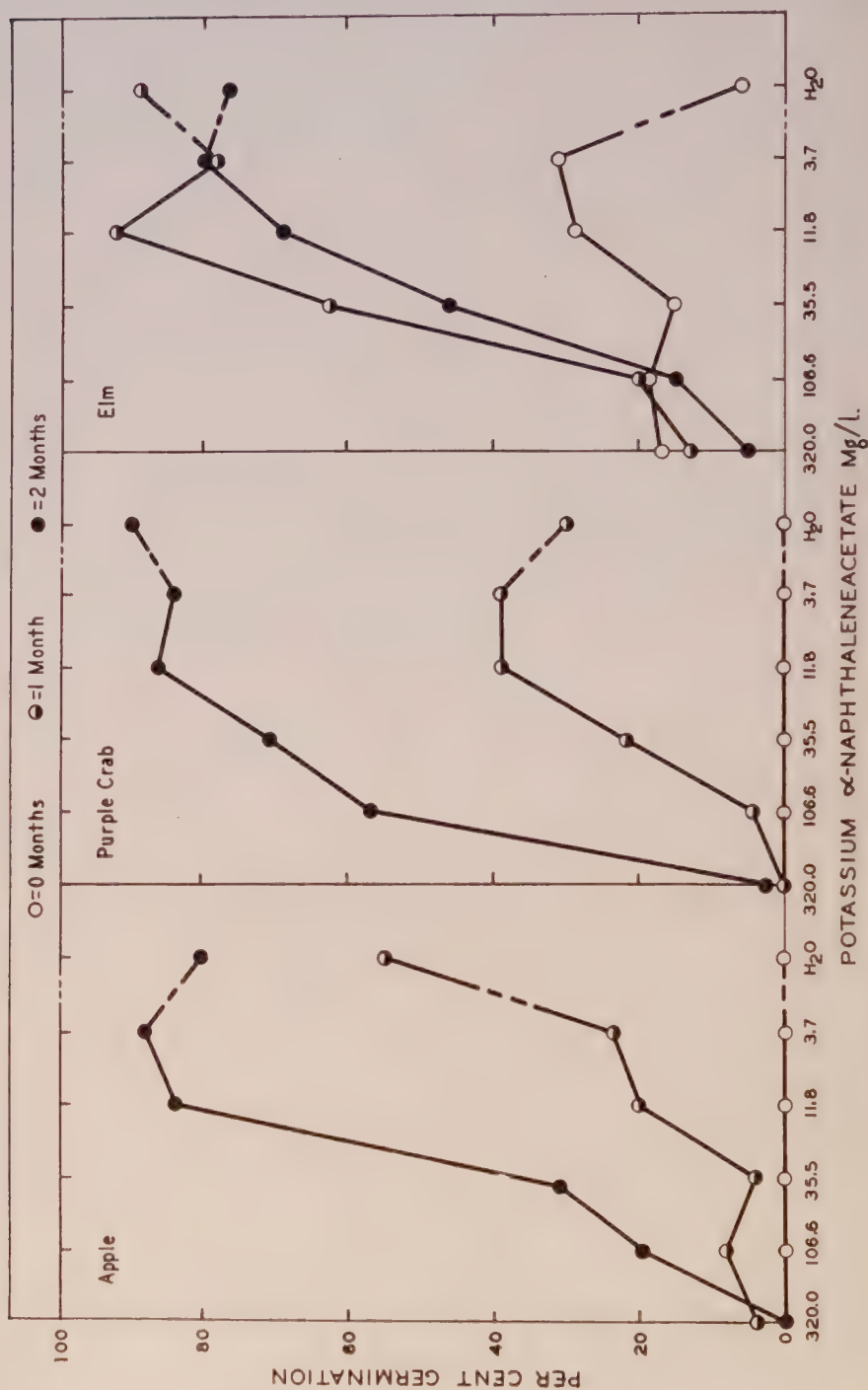


FIGURE 2. The effect of soaking seeds in growth substance solutions after 0, 1, or 2 months of stratification at 5° C.

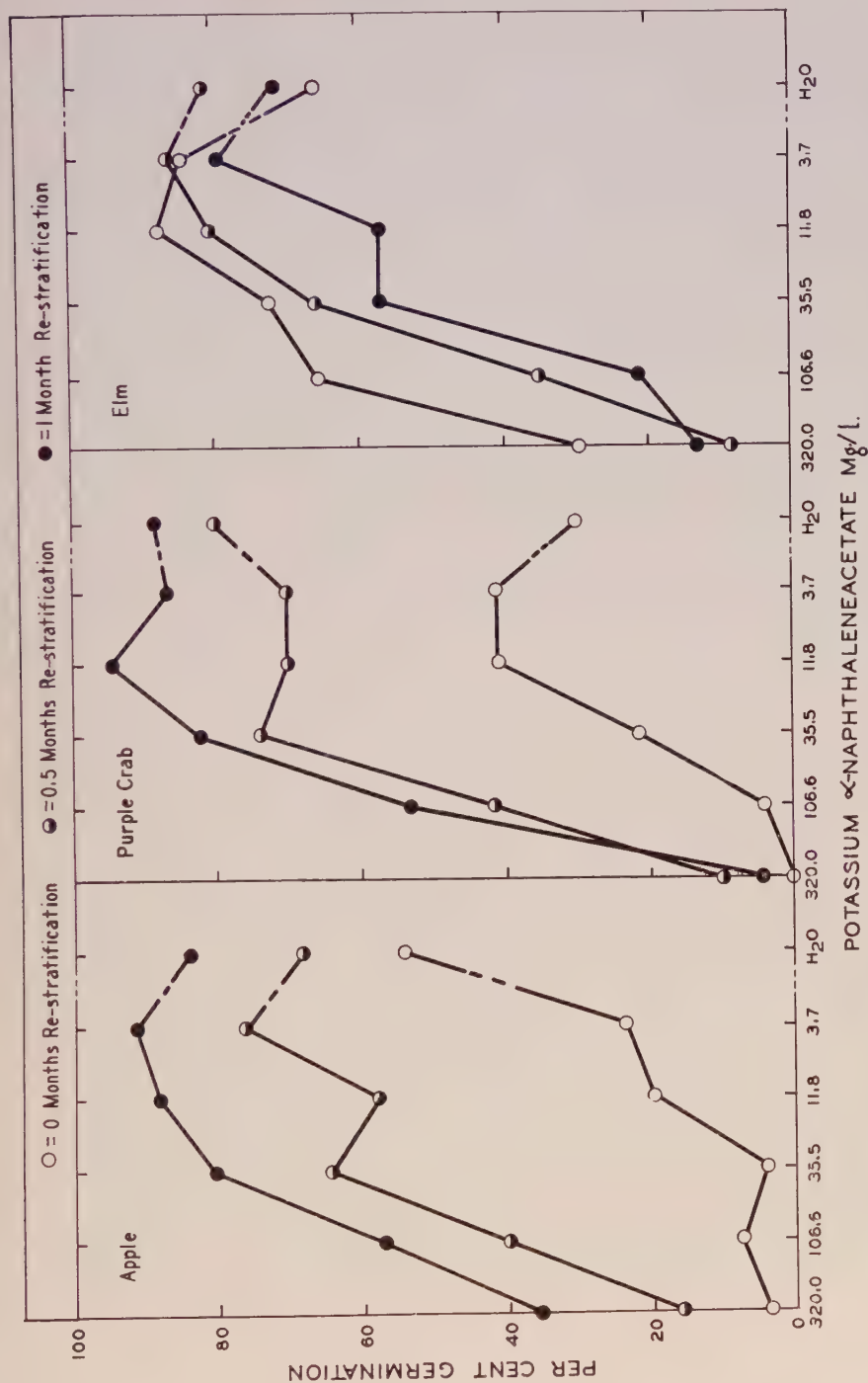


FIGURE 3. The effect of soaking seeds in growth substance solutions after stratification for one month (apple and Purple crab apple), or one-half month (elm), at 5° C., followed by a re-stratification period at 5° C. of zero, one-half, or one month before planting.



FIGURE 4. The effect of 3 mg. l. solution of  $\beta$ -indoleacetic acid on excised apple embryos. Left, Untreated. Right, Treated. A, Non-after-ripened embryos. B, After-ripened embryos.



which were obtained from The Kilgore Seed Company at Plant City, Florida, and were received in this laboratory on October 10, 1939. A number were shelled immediately and soaked for 24 hours at 20° C. in solutions of potassium  $\alpha$ -naphthaleneacetate of varying concentrations and in water after which the seeds were mixed with moist granulated peat moss and placed at 20° C. for germination. The solutions were ineffective in increasing the germination percentage of these seeds which were only partially dormant, since the control lot germinated 36 per cent. Dust treatment of the seeds of this lot with Rootone (1939), Merck powder preparation containing 5 mg./g. indolebutyric acid, or talc in which the seeds were mixed with the dust and the excess powder sieved off seemed to inhibit germination regardless of whether the dust contained a growth substance. Dry seeds behaved in the same manner as those soaked in water, so that the soaking effect could be disregarded in this experiment.

When the second test of these seeds was made 21 days later, the partial dormancy exhibited by the initial test had been completely overcome in dry storage. The results here simply indicated that none of the treatments applied was toxic. Further tests will be made when fully dormant peanut seeds can be obtained.

Several experiments were also performed using non-after-ripened and after-ripened excised embryos of the apple. These data will not be presented in detail since they all show similar effects to those pictured in Figure 4. The non-after-ripened embryos when treated with growth substances showed more growth within a certain time period than the control lot. This is seen in Figure 4 A. However, it will be noted that this growth is abnormal. Most of these seedlings when transferred to soil in pots failed to survive.

The effect of an after-ripening period of six weeks prior to treatment is shown in Figure 4 B. Here the abnormal growth of seedlings treated with growth substance as compared with the control lot is again evident.

Growth substance treatments given to germinated seeds of tree peony, *Viburnum*, and *Lilium auratum*, all of which exhibit dormancy in the shoot or the bud which forms it, failed to have any effect on the dormancy and in many instances killed the seedlings.

#### SUMMARY

Treatment of dry, dormant seeds of the domestic apple (*Pyrus* sp.) and *Pyrus malus* var. *niedwetzkyana*, with growth substances in concentrations of 3.7 to 320.0 mg./l. had no effect on their subsequent production of seedlings. On the other hand, seeds of *Ulmus americana*, which did not require a pretreatment period at low temperature for germination, but which showed improved germination capacity with such treatment, seemed to benefit by the application of solutions of 35.5, 11.8, or 3.7 mg./l. of potas-

sium  $\alpha$ -naphthaleneacetate. This benefit was relatively small, however, so that growth substance treatment of these seeds could not be used as a substitute for either low-temperature pretreatment or soaking in water under a light source as a means of speeding up the germination.

The germination of seeds of *Cornus florida*, *Cornus stolonifera*, *Pyrus* sp., and *Pyrus malus* var. *niedwetzkyana*, which had been after-ripened by a period at 5° C., was inhibited by treatment with growth substances. This inhibiting effect was partially removed by a second period at 5° C., but abnormalities often appeared in the seedlings so produced.

Growth substance treatment of germinated seeds of *Lilium auratum*, *Paeonia suffruticosa*, and *Viburnum* sp. failed to initiate growth of the dormant epicotyls.

## FEEDING TESTS WITH THIOUREA (THIOCARBAMIDE)

FREDERICK B. FLINN<sup>1</sup> AND JOHN M. GEARY<sup>1</sup>

Experience has emphasized the necessity of studying the possible toxicity of any chemical compound with which human beings may come in contact. This is particularly true when the compound may be inhaled or ingested by the exposed person. A study of the chronic effects produced by the continual intake of small amounts is more important from a public health viewpoint than the acute toxic or sub-acute toxic effects caused by large doses.

The suggested use (1, 2) of thiourea,  $\text{NH}_2\text{CSNH}_2$ , also called thiocarbamide, to prevent the browning of cut surfaces of fruits and some vegetables has necessitated such a study. This is particularly essential as it has been found that the minute amount of thiourea present must remain on the cut surface to prevent the browning action.

On account of the possibility that the thiourea method which has been proposed might find application in the food industry, Boyce Thompson Institute for Plant Research, Inc. requested that a study be made of the toxicity of this chemical, in order to determine whether the ingestion of it in food would be injurious to health.

The method of treating the fruit is to dip the sliced, diced, or otherwise cut fruit in a 0.10 per cent solution of thiourea. More recent tests indicate that a 0.05 per cent solution of thiourea is sufficient to protect the fruit. Experiments have shown that a kilogram of sliced apples upon being dipped takes up 60 cc. of the thiourea solution, or 30 milligrams of the chemical. From these data one arrives at the figure of 0.086 milligram per kilogram of body weight as the amount a man with body weight of 70 kilograms would ingest if he ate 200 grams of treated fruit.

The results of the feeding tests are reported herewith.

### EXPERIMENTAL STUDIES

Rabbits and albino rats were selected for the purpose of determining the acute and sub-acute toxic doses. The rabbits used in this work weighed approximately two kilograms each; the rats from 225 to 250 grams each.

Thiourea was dissolved in tap water and given the rabbits by means of a stomach tube. For the rats it was dissolved in physiologic saline solution and injected subcutaneously. In one series of experiments the thiourea was injected into the tail vein of a rat.

Groups of rabbits given up to 9 grams of thiourea per kilogram of body weight by means of a stomach tube had no fatalities. They ate, looked, and

<sup>1</sup> Delamar Institute of Public Health, Columbia University, New York, N. Y.

behaved in the same manner as the control group. Perhaps the groups receiving the high amounts drank more water than the control group or those receiving smaller amounts.

In the groups of rabbits getting 10 grams of thiourea per kilogram of body weight, 50 per cent died. The animals were quiet and the respiration changed in type and rate. The respiration was deeper and more labored. In the case of the animals which survived, this condition passed off in four or five hours. The first death in the group occurred eight hours after the animal had received the dose. The death in the case of the other two did not occur until the following day.

The mortality rate was 100 per cent when 11 grams per kilogram of body weight were administered in a 20 per cent water solution (after increasing the temperature of the water to increase the solubility of the chemical). The first rabbit died in seven hours and the last death occurred at the end of 36 hours. Autopsy revealed an edematous condition of the lungs. The liver, spleen, and kidneys were normal. The stomach generally showed several areas of congestion.

Rats given the same doses per kilogram of body weight subcutaneously in saline solution paralleled the results shown by the rabbits.

The behavior of the group of rabbits receiving the 11 grams per kilogram of body weight led one to feel that the distress was due to some physical reaction in the animal rather than to any true chemically toxic action. It must be remembered that the animal received 220 cc. of water containing 22 grams of thiourea at one time in this experiment. It was observed that the thiourea tended to crystallize out unless the solution was slightly warm. It even tended to crystallize out while passing through the stomach tube. One was led to believe that the death was due to a physico-chemical action such as osmotic pressure. For this reason the present authors repeated the experiment of Molitor (3) by giving 200 cc. of a solution of sodium chloride having the same osmotic pressure as the thiourea or 7.6 per cent NaCl. The rabbits all died within 24 hours. Rabbits given 200 cc. of water in one dose by means of a stomach tube all survived. Because the 7.6 per cent NaCl solution caused 100 per cent fatality and the animals behaved in the same manner as those receiving thiourea, one is justified in assuming that there is no danger of acute poisoning among human beings from oral ingestion of thiourea. It might be said that it is non-toxic in the practical sense of the term.

Six rats four months old and weighing on the average 250 grams were given 6.3 milligrams of thiourea in 0.20 cc. of physiologic saline solution intravenously through the tail vein. Three of the rats died within 24 hours. The first effect noted was a labored breathing and condition of shock similar to that which is observed when a foreign protein is injected in the same manner. Toward the end, the animal had a slight convulsion. When the



animal was autopsied the only change which could be detected was marked edema of the lungs. The animals which survived showed the same condition of shock and labored breathing for three or four hours; then followed a gradual return to normal, and the labored breathing passed away.

It has never seemed a fair test to inject a substance intravenously when the only method of ingestion in the case of a food product would be by mouth or lungs. We have observed that when other substances are injected intravenously the type of reaction was entirely different from that resulting from oral ingestion. For example, copper salts will lake the blood but when given by mouth no such reaction takes place even with much larger doses.

#### EFFECTS OF DAILY INGESTION OVER A PERIOD OF WEEKS

Four dachshund puppies three weeks old were used in part of this experiment. For two or three weeks their diet consisted chiefly of "Ween," a canned milk product containing all of the necessary ingredients required for growth. In addition they were given a teaspoonful of cod liver oil. At the end of three weeks they began to receive raw meat. Their daily diet was as follows: milk in the morning, raw chopped meat at noon, and "Grow-pup" at night.

TABLE I  
SHOWING THE INCREASE IN WEIGHT OF DACHSHUND PUPPIES  
(FOUR PUPPIES IN TEST)

Date	Av. weight in ounces
Feb. 13	44
" 20	50
" 27	59
Mar. 6	70
" 14	81
" 20	92
" 27	100
Apr. 3	No weights taken as puppies were wormed
" 11	107
" 15	129
" 17	140
" 21	140*

\* The av. weight of controls was 139.

They were given 25 milligrams of thiourea per kilogram of body weight daily. Until they began to receive meat, it was administered in the milk. Afterwards it was mixed with their meat. The dogs were kept under observation until they had consumed all of the food placed in front of them to insure that they were ingesting the thiourea.

They were kept on the above diet for ten weeks or until the owner who had loaned the litter to us requested their return. The owner kept the control group at home on the same diet. Throughout the entire experiment the

puppies receiving the thiourea grew in a normal manner, were very active all the time, and the condition of their hair indicated a general healthy condition. Everyone who saw them noted the fine appearance and behavior of the group. Their weight was slightly above the control dogs. Table I shows the increase in weight during the course of the experiment.

*Rabbits.* The rabbits used in the work were fed hay and "Purina" rabbit chow. Water was given at all times. Eight rabbits weighing around two kilograms each were used in the exposed group, and four in the control group. Each animal in the exposed group was given 25 milligrams of thiourea per kilogram of body weight every day except Sundays; the thiourea was dissolved in water and administered by mouth by means of a pipette.

The rabbits remained healthy and active during the entire 12 weeks that they were being observed. They increased in weight and compared very favorably with the control group (Table II).

TABLE II  
SHOWING THE INCREASES IN WEIGHT IN RABBITS (8 IN THE EXPOSED  
GROUP AND 4 IN THE CONTROL)

Date	Av. weight in ounces, exposed rabbits	Av. weight in ounces, control rabbits
Feb. 13	68	64
" 20	63	63
" 27	66	65
Mar. 6	79	66
" 14	71	69
" 20	69	67
" 27	72	70
Apr. 3	69	60
" 11	78	75
" 17	78	74
" 25	83	78
May 2	86	78
" 8	85	74

At the end of the exposure they were sacrificed for examination. A few areas of coccidiosis were observed in the liver. Otherwise the gross pathologic examination was negative. Dr. W. C. VonGlahn, Associate Professor of Pathology, College of Physicians and Surgeons, Columbia University, kindly examined the slides for us and saw no changes in the tissues. A typical finding was as follows:

Lungs normal.

Kidney normal.

Liver normal.

Spleen very small amounts of hemosiderin in the phagocytes. "No lesions can be found in these tissues."

*Rats.* Thirty-four rats six weeks old were used for this work. Twenty-two were placed in the exposed group and 12 were used as controls. The

average weight of the rats in the exposed group at the beginning of the test was 133 grams as compared with 155 grams in the control group. The rats were fed on chicken laying mash and were given water at all times. Because of the difficulty in giving such a large group the thiourea orally by means of a stomach tube or pipette, it was decided to give it to them in their drinking water. We had found from previous work that the rat under normal conditions as to temperature drank approximately 15 cc. of water per day. We started to give each rat 25 milligrams of thiourea per kilogram of body weight in this quantity of water. Shortly after the experiment had started it was found that the rats were drinking 40 cc. daily per rat. We decided not to change the amount of thiourea per cc. of water but to determine how the rat would flourish on 60 milligrams of the compound per kilogram of body weight as compared with the dogs and rabbits which were receiving 25 milligrams per kilogram of body weight.

TABLE III  
SHOWING THE INCREASE IN THE WEIGHTS OF RATS (22 IN THE EXPOSED  
GROUP AND 12 IN THE CONTROL)

Date	Av. weight in grams, exposed group	Av. weight in grams, control group
Feb. 13	133	155
" 20	141	167
" 27	153	179
Mar. 6	150	180
" 14	174	201
" 20	173	202
" 27	175	203
Apr. 3	177	215
" 11	188	216
" 17	192	218
" 25	197	223
May 2	197	230
" 8	205	237

Inasmuch as the control group of rats drank the same amount of water as the exposed group, one cannot ascribe the extra amount of water consumed to the ingestion of thiourea.

The increase in body weight in the exposed group was more gradual as compared with the control group but the percentage gain at the end of the experiment was found to equal that of the control group (Table III). We have observed that rats are quite sensitive to disagreeable tastes and the liquid which they drank had a slightly bitter taste. Some of the rats were killed at the end of the experiment and examined. Only a gross examination was made, as the organs showed no changes that were not present in the control group.

The doses given the rats would be comparable to a dose of 4.2 grams per day for a man weighing 70 kilograms. In the case of the dogs and rabbits

the corresponding dose for man would be 1.75 grams. These amounts are 290 to 700 times the amount that a man would ingest when eating 200 grams of treated fruit. The dose for rats and rabbits giving 50 per cent fatalities is 10 grams per kilogram, which would be equivalent to 700 grams for a man weighing 70 kilograms.

#### SUMMARY

Groups of rabbits given 9 grams of thiourea (thiocarbamide),  $\text{NH}_2\text{-CSNH}_2$ , per kilogram of body weight by means of a stomach tube, showed no fatalities, but the animals ate, looked, and behaved in the same manner as individuals in the control group.

In groups of rabbits receiving 10 grams of thiourea per kilogram of body weight, 50 per cent of the animals died.

The mortality rate was 100 per cent when 11 grams per kilogram of body weight were administered.

Albino rats receiving subcutaneous doses of thiourea equal on the basis of the body weight to those given to rabbits by the stomach tube, showed results which were parallel to those obtained in the rabbit tests; that is, the toxic limits were the same for the two species.

The introduction into the stomachs of rabbits of a solution of sodium chloride at a concentration of 7.6 per cent (which has the same osmotic pressure as the fatal dose of thiourea) also caused 100 per cent fatality, and the animals behaved in the same manner as those receiving the thiourea solution. Dachshund puppies which were kept on a diet containing thiourea in amounts equal to 25 milligrams per kilogram of body weight for 10 weeks, showed no injurious effect, were lively in behavior, and gained in weight in a normal manner.

For a period of 12 weeks rabbits were given daily, except Sunday, by the mouth an aqueous solution containing thiourea in amounts to give 25 milligrams of thiourea per kilogram of body weight. Their weight increase did not differ materially from that of the controls.

During nearly 12 weeks rats were given thiourea in their drinking water in amounts equal to 60 milligrams per kilogram of body weight per rat. The weight gain was more gradual in the exposed group as compared with the controls, but the percentage gain at the end of the test was the same for the two groups.

Autopsies on rats and rabbits receiving these small doses of thiourea revealed no lesions in any of the tissues examined.

Since it is estimated that a kilogram of sliced apples treated with a 0.05 per cent thiourea solution retains 30 milligrams of thiourea upon its surfaces, the amount of thiourea ingested in eating 200 grams of the fruit would be 6 milligrams, or approximately 0.086 milligram per kilogram of body weight for a 70-kilogram person. The daily doses given to rats with-



out any unfavorable effects over a period of approximately 12 weeks would be comparable to a dose of 4.2 grams per day for a person weighing 70 kilograms. In the case of the dogs and rabbits the corresponding dose for man would be 1.75 grams. These amounts are about 300 to 700 times the amount ingested by a man eating 200 grams of treated fruit.

The toxic dose for rats and rabbits is about 10 grams per kilogram of body weight, which would be equivalent to about 700 grams of thiourea for a man weighing 70 kilograms. This amount is more than 100,000 times the amount that would be ingested in eating 200 grams of fruit treated with thiourea.

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## PRELIMINARY FEEDING EXPERIMENTS WITH THIOUREA (THIOCARBAMIDE)<sup>1</sup>

ALBERT HARTZELL

Previous experiments (2) have shown the effectiveness of small amounts of thiocarbamide, also called thiourea,  $\text{NH}_2\text{CSNH}_2$ , in preventing the browning of cut tissues such as those involved in the drying of fruits and vegetables, in the preparation of fresh fruit salads, in the canning of various fruits, or in their use in the pie-baking industry. Experiments were undertaken, therefore, to determine whether thiocarbamide was at all toxic when used in the quantities likely to be obtained in the consumption of food treated with it, and if not, whether it could become toxic if eaten in higher amounts.

Little has been published on the tolerance of higher animals and man to thiourea, particularly in the way of feeding experiments.

Lange (6) administered 2 g. of thiourea in a gelatine capsule into the stomachs of a rabbit and a dog without injurious effects. A similar amount in a physiological salt solution was injected into the jugular vein of a rabbit. After recovering from the seizure which occurred almost immediately it showed no appreciable symptoms. At the beginning of the application the pulse and respiration rate rose perceptibly but within an hour returned to normal. He proved by urinalysis that thiourea administered by the mouth is taken up into the circulation soon after feeding (at least after three hours).

Binet (1) found that the fatal dose for the green frog is about 0.10 g. per 10 g. (10 g. per kg.) of animal and reports that he obtained very appreciable effects with moderately weak doses but no data are given on these weak doses. If the dose is not fatal the motor inertia is gradually dissipated; the movements are at first weak accompanied by slight ataxic twitchings of the muscles. Gradually the animal returns to its normal aspect. With increased dose, the beating gradually slows down and the heart stops in diastole. The heart remains excitable for a certain time, but the diastole is not renewed under the influence of atropine, as is the case with poisoning with muscarine. The toxic dose for the guinea pig is about 0.40 g. per 100 g. (4.0 g. per kg.) of animal. In warm-blooded animals (guinea pig and rat) subcutaneous injections of thiourea cause weakness with progres-

<sup>1</sup> The writer is indebted to Dr. F. R. Weedon, formerly Assistant Director, Bureau of Laboratories, Department of Health, Yonkers, New York, and Pathologist of Yonkers Professional Hospital, and now Director, Jamestown Municipal Laboratory, Jamestown, New York and Director, Chautauqua County Laboratory, Dunkirk, New York for guidance in the medical phases of this investigation.

sive coldness ending in coma and death, without convulsions. Shortly after a peritoneal injection, the animal presents the phenomena of appreciable morbidity, feebleness, and trembling. The temperature gradually falls, heart beat diminishes, becomes irregular and slows down. The sensibility does not appear to be affected. The sensorium is not engorged as with urethanes. The animal remains several hours in this state, and gradually recovers, if the dose is not too great. If it is fatal, the animal becomes cold with the heart beat feeble and then falls in collapse, remains immovable on the side, and from time to time twitching of the feet occurs. The respiration remains superficial, the heart beat barely perceptible, and death gradually occurs without convulsions. The urine produces a copious precipitate of sulphur with the addition of lead acetate and soda. It also darkens in Fehling's solution. The blood does not seem to be altered either spectroscopically or otherwise. With mammals, to recapitulate, Binet (1) states that death occurs in collapse with gradual coldness, without convulsions.

Knapp (4) extirpated the kidneys from a rabbit and injected thiourea (amount not stated) subcutaneously and intravenously and found thiourea in the filtrate of the intestinal contents. He calls thiourea a physiologically indifferent substance.

Kastle and Elvove (3) introduced 0.5 g. of thiourea subcutaneously into a guinea pig. At first the pig exhibited greater distress than one which received an equal amount of ammonium sulphocyanate. At the end of an hour it was observed to be in a semi-comatose condition. It completely recovered overnight and remained normal and active until the end of the experiment. The urine from this animal contained an abundance of thiourea, but no sulphocyanate. He concludes that these two compounds remain unaltered in the animal organism.

Pohl (10) reports that if 1 to 2 g. of thiourea is given to dogs, cats, or rabbits by way of mouth, subcutaneously, or intravenously there is a strong leek or radish odor which lasts for hours or even for days. Most of the thiourea goes into the urine unchanged. He regarded thiourea as entirely non-toxic.

Sato (11) injected subcutaneously 0.3 g. of thiourea in a 10 per cent aqueous solution into a rabbit. The appetite was poor and the animal appeared to be sick following the injection and on the next day, but had fully recovered three days after the injection, at which time it was again injected with 0.5 g. thiourea in 5 cc. of sterile water. He found that this dose had no noteworthy toxic effect.

Masuda (7) repeated Sato's experiments and reports the same general conclusion. His animals were fed cauliflower, a high sulphur-containing food product.

Kojo (5) introduced 3 g. of thiourea into a dog by way of mouth two days in succession and observed no injurious effects, but when on the third



day 3 g. were injected subcutaneously into the same animal it became ill but recovered on the third day following. No illness followed a second subcutaneous injection of 3 g. a week later.

Nicolas and Lebduska (9) found that 1 g. of thiourea per kg. of body weight introduced into the duodenum of a dog did not increase the blood pressure or influence the rate of respiration. They report that thiourea is less toxic than urea to dogs and rabbits, the lethal dose for the latter with subcutaneous injections being 10 to 11 g. per kg. of body weight.

Medes (8) reports symptoms of prostration in a human who had ingested 0.82 g. of thiourea. She found 6.36 mg. of thiourea<sup>2</sup> injected intravenously fatal to rats. Experiments on the oxidation of sulphur-containing compounds (8) in the body showed that sulphur to account for all of the thiourea is completely excreted in the urine within 48 hours. Ninety-two per cent of the recovered sulphur was in organic form. Along with thiourea she states there was an increase in cystine.

Wood and others (12, p. 1628) in the United States Dispensatory, considered thiourea "a very active poison, paralyzing the nerve centers, but leaving intact the peripheral nerves and muscles." They cite Paul Binet's work (1) on thiourea as proof for this statement.

It will be noted that in nearly all of the experiments in which toxicity of thiocarbamide was noted the chemical was injected into the animal either subcutaneously or intravenously. While such methods are suitable for experiments on a study of the metabolism of the chemical (which was the sole purpose in most of the experiments) and give information on the toxicity of the chemical under the conditions of the test, it seems clear that experiments to determine the suitability of food treated with thiocarbamide for consumption by animals should be based on feeding experiments. Of these there are very few tests reported in the literature and in these cases no injurious effect was noted except in one case, that of Medes (8).

#### FEEDING TESTS ON GUINEA PIGS

In this study, which was made at the Boyce Thompson Institute for Plant Research, Inc., preliminary tests were made on guinea pigs. Ten males ranging in age from 26 to 28 weeks were confined in groups of two in cages made of galvanized iron and wire. Pine shavings were used as bedding. The ration consisted of treated cabbage leaves and untreated whole oats (Table I) which was offered slightly in excess of what the animals would eat for a period of six weeks. Four control animals were fed untreated oats and cabbage. The thiourea compound to be tested was dissolved in distilled water and the solution sprayed on the cabbage leaves by means of an atomizer and allowed to evaporate at room temperature before the

<sup>2</sup> Private communication.

leaves were offered for food. Sprayings were made daily so that the residue would deteriorate as little as possible before being eaten. The results are shown in Table I and Figure 1.

Two guinea pigs (Fig. 1 B) fed on 0.1 per cent thiourea-treated leaves showed an average gain in weight of 146.5 g. A total of 135 cc. of an aqueous solution of thiourea (0.1 per cent) was used to treat the cabbage leaves consumed by this pair. Another pair (Fig. 1 C) fed 1.0 per cent thiourea-treated leaves showed an average gain in weight of 121.5 g. A total of 137 cc. of an aqueous solution of thiourea (1.0 per cent) was used to treat cabbage leaves consumed by these two animals. Two controls (Fig. 1 A) which were given a ration of untreated cabbage and oats showed an average gain

TABLE I  
RECORD OF WEIGHT OF GUINEA PIGS FED CABBAGE LEAVES TREATED  
WITH THIOUREA AND UNTREATED OATS

Guinea pig No.	Conc. thiourea, %	Wt. at beginning of exp. Nov. 8, grams	Wt. at end of 2nd week, grams	Wt. at end of 4th week, grams	Wt. at end of 6th week, grams	Av. consumption oats per animal, grams	Av. gain in wt., grams
1	0.1	539	610	664	688	1200	146.5
1'	0.1	456	541	610	600		
2	1.0	537	622	674	663	1050	121.5
2'	1.0	512	579	621	629		
Ck.	—	520	552	622	648	1125	97.5
Ck.'	—	600	613	608	667		
3	3.03*	530	542	573	587	1125	57.0**
3'	3.03*	432	361	—	—		
Ck.	—	466	532	547	588	1150	128.5
Ck.'	—	440	507	534	572		

Note:—The upper series was begun Nov. 8; lower series Nov. 22, 1935.

\* Refused to eat leaves sprayed with sat. and 50% sat. solutions.

\*\* Guinea pig 3' died of infection Dec. 8.

in weight of 97.5 g. One guinea pig (Fig. 1 E) fed on 3.03 per cent thiourea-treated leaves gained 57.0 g. in weight. Another guinea pig similarly treated died 16 days after the beginning of the experiment. This animal was submitted to Dr. Ward H. Cook of the Yonkers Bureau of Laboratories who pronounced the cause of death as due to infection. The total amount of aqueous solution of thiourea (3.03 per cent) used to treat cabbage leaves consumed by these two guinea pigs was 95 cc. Two control animals (one shown in Fig. 1 D) which were fed a ration of untreated cabbage and oats showed an average gain of 128.5 g. The treated cabbage in all cases was readily accepted. No irritation of the eyes or skin was noted in any of the animals fed treated leaves. No symptoms of paralysis were noted. Both treated and controls appeared normal in neuro-muscular responses. As

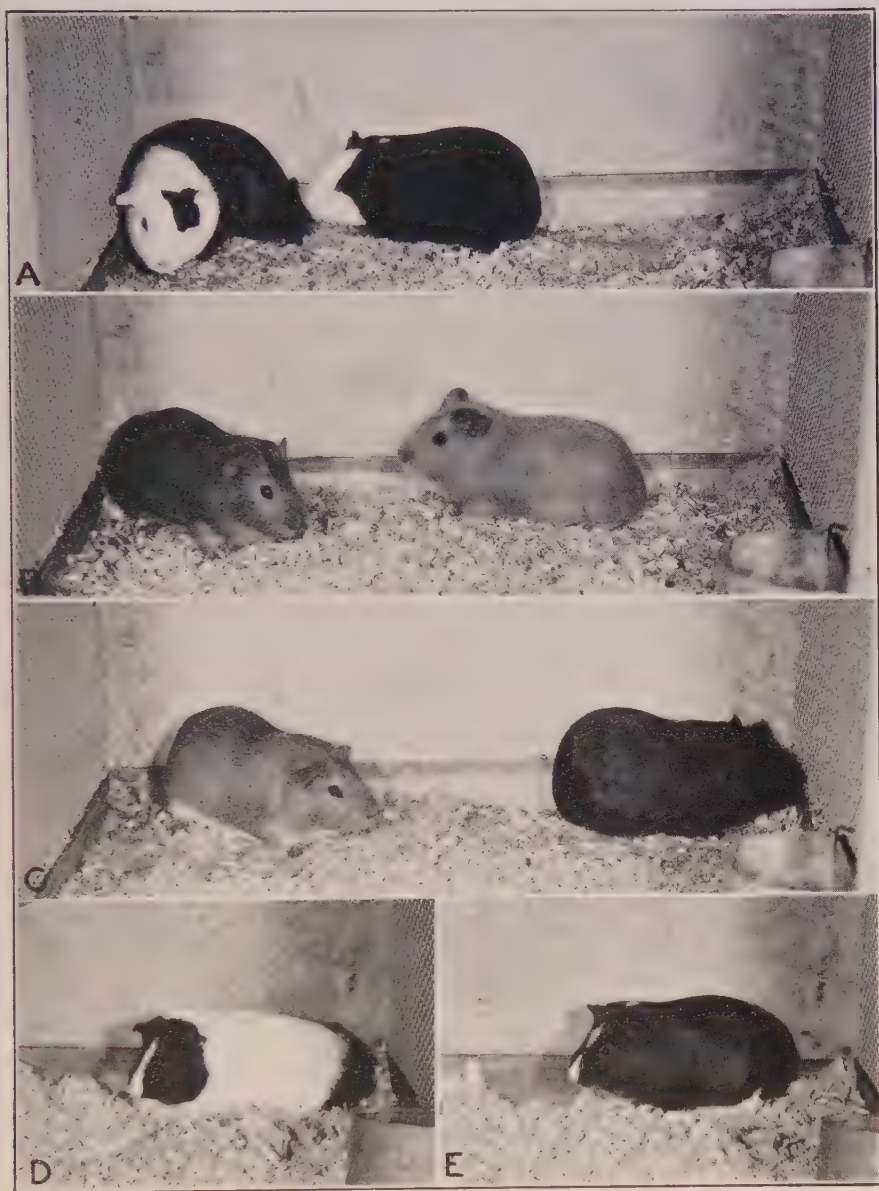


FIGURE 1. Guinea pigs used in feeding experiments photographed at the end of the six-weeks period. A. Control; B. Pair fed thiourea-treated cabbage (0.1%); C. Pair fed thiourea-treated cabbage (1.0%). D. Control; E. Guinea pig fed thiourea-treated cabbage (3.03%). This animal (E) consumed approximately 45 mg. of thiourea per kg. of body weight per day. All animals appear normal at the end of the experiment.



the toxic dose of the residues ingested was doubtless below the lethal dose, no autopsies were performed except on the individual that died of an infection.

Saturated solutions and 50 per cent saturated solutions of thiourea were sprayed on cabbage leaves but the guinea pigs refused to feed on them.

#### HUMAN FEEDING TEST

Two apples having a total weight of about 280 g. were sliced and dipped in 0.1 per cent thiourea solution. The sliced apples were dried overnight at room temperature. Using 0.1 per cent thiourea solution, each 100 g. of sliced apples take up 0.006 g. of thiourea. Therefore 280 g. of sliced apples would contain 0.0168 g. of thiourea. Slices of these treated apples were eaten January 14 and 15, 1936. The treatment did not impair the palatability. The writer experienced no discomfort from eating these treated apples and noticed no symptoms of poisoning. Half the concentrations used would have protected the apple tissue from oxidation.

#### FEEDING TESTS WITH RATS

Additional feeding tests were made using the rat as the test animal. As a preliminary test, one rat was caged separately and fed 7.5 mg. of thiourea per day in milk, which it readily accepted. When this concentration (7.5 mg. per rat per day) was offered to a group of six rats they refused to accept it when the thiourea was added to milk. However, by adding 4.9 mg. of thiourea to each 25 cc. of the drinking water supplied to each rat per day the chemical was accepted readily by the rats. Forced feeding was not attempted.

After these preliminary tests were completed, 24 young mature male rats were divided into four groups each of six individuals. The general arrangement and results of this experiment are shown in Table II. A quarantine period of two weeks preceded the starting of the feeding tests. The groups were confined in cages made of galvanized iron and wire. Pine shavings were used as bedding. An inverted box in each cage provided with an opening at each end afforded a place for the animals to hide. The ration consisted of Beacon dog pellets,<sup>3</sup> water or milk which were supplied continuously. This was supplemented with a weekly ration of lettuce.

The rats in cage 1 were fed thiourea in their drinking water in amounts equal to 4.9 mg. of thiourea per rat per day. Each rat in cage 2 was fed daily 10 cc. of milk plus 2 cc. of an aqueous solution containing 0.0363 mg. of thiourea. The controls were in cages 3 and 4. It will be noted in Table II that the average gain in weight for the treated animals was comparable to that made by the controls.

<sup>3</sup> The manufacturer's guarantee as stated on the container follows: protein, not less than 24%; fat, not less than 3%; fiber, not more than 5%; carbohydrates, not less than 50%.



TABLE II  
RECORD OF WEIGHT OF RATS FED THIOUREA

Cage No.	Mg. thiourea per kg. of body weight per day	Rat No.	Amt. of thiourea per rat per day, mg.	Wt. at beginning of exp. Apr. 5, 1939, grams	Wt. at end of first week, grams	Wt. at end of second week, grams	Wt. at end of third week, grams	Wt. at end of fourth week, grams
Rats fed thiourea for 22 days								
1	21.6	1	4.9	189	198	190	198	193
		2	4.9	137	161	167	183	181
		3	4.9	147	159	161	173	168
		4	4.9	171	180	172	180	178
		5	4.9	150	169	168	185	183
		6	4.9	187	200	189	203	202
		Av. wt.		163.5	177.8	173.5	187.0	184.1
Rats fed thiourea for 28 days								
2	0.19	1	0.0363	170	191	197	208	210
		2	0.0363	137	147	155	166	170
		3	0.0363	171	184	182	191	195
		4	0.0363	151	171	181	200	210
		5	0.0363	141	155	161	174	181
		6	0.0363	138	127	103	95	*
		Av. wt.		151.3	162.5	163.1	172.3	193.2
Controls fed milk								
3	0	1	—	140	158	166	170	171
		2	—	205	220	222	235	238
		3	—	201	220	224	232	239
		4	—	124	143	152	164	127
		5	—	139	129	139	154	159
		6	—	141	164	172	180	185
		Av. wt.		158.3	172.3	179.1	189.1	186.5
Controls not fed milk								
4	0	1	—	207	212	203	212	219
		2	—	172	180	185	196	193
		3	—	204	208	206	217	223
		4	—	190	**	—	—	—
		5	—	179	172	163	*	—
		6	—	190	189	195	200	205
		Av. wt.		190.3	194.0	190.4	206.1	210

\* Died April 27, 1939 of an infection.

\*\* Removed from experiment because of an infection.

When 1000 g. of plant tissue, such as sliced apples, are dipped into a 0.05 per cent thiourea solution, approximately 60 cc. of the liquid are taken up by the tissue. The amount of thiourea in 60 cc. of a 0.05 per cent thiourea solution is 30 mg. and the amount taken up by 200 g. of tissue is 6

mg. Therefore, the amount of thiourea ingested by a 70 kg. man in eating 200 g. of treated fruit would be  $6 \div 70$ , or 0.086 mg. per kg. of body weight.

Therefore, the rats in cage 1 receiving 4.9 mg. of thiourea per rat per day, or 30 mg. per kg. of body weight, were receiving more than 300 times

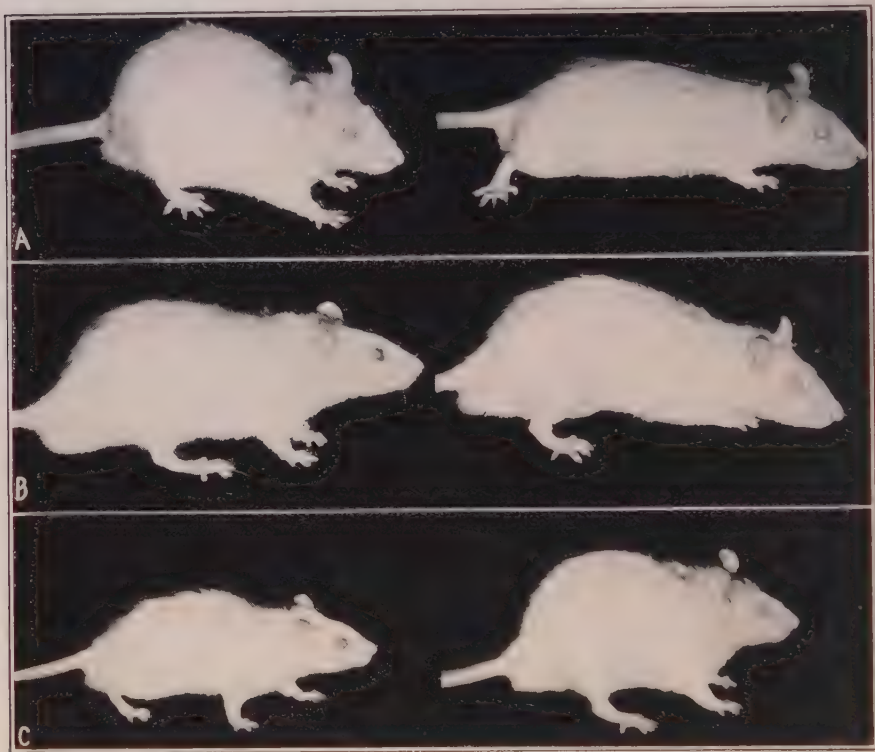


FIGURE 2. Rats used in feeding experiments photographed at the end of the four-weeks period. In each case, control on the left, treated on the right. A (right). Rat which consumed 21.6 mg. of thiourea per kg. of body weight per day. B (right). Rat fed milk. C (right). Rat fed 49 mg. of thiourea per kg. of body weight per day photographed at end of the 22-day period. All animals appear normal at the end of the experiment

the amount of chemical which would be ingested by a person of 70 kg. body weight, consuming 200 g. of fruit tissue treated with thiourea to prevent browning.

None of the rats fed the highest concentration died during the course of the experiment. One rat in cage 2 which was fed the lowest amount of thiourea died after being in the experiment 22 days. It was submitted immediately for autopsy by Dr. F. R. Weedon of the Bureau of Laboratories of Yonkers, New York, who reported the cause of death as due to an infection.

Two control animals showing symptoms similar to the above animal were removed during the course of the experiment to avoid infection of the other animals. Aside from the three animals eliminated because of an infection, all animals appeared to be normal (Fig. 2 A and B).

#### RAT FED 49 MG. PER KG. PER DAY

A young mature male rat was caged separately and fed milk containing 7.5 mg. of thiourea per day for 27 days (from April 5, 1939 to May 2, 1939). A total of 202.5 mg. of thiourea was consumed by this individual. During the period of the experiment the rat increased in weight from 123 g. to 153 g. The dose received by this animal averaged 49 mg. per kg. of body weight per day. This was 570 times a man's dose (allowing 0.086 mg. per kg. per day as a man's dose). A control animal caged separately showed a gain in weight from 162 g. to 195 g. Both animals appeared to be normal at the end of the experiment (Fig. 2 C).

#### RAT INJECTED WITH THIOUREA

A rat was injected at noon, February 22, 1939, with 1 cc. of 10 per cent thiourea (0.075 g.) in physiological salt solution intravenously into the tail vein. About 0.25 cc. went into tissue outside the vessel. The rat was under ether anaesthesia. During recovery from the anaesthetic the hind legs seemed weak for about five minutes. No other symptoms were observed. The animal was found dead at 9:00 a.m., February 24, 1939. Autopsy showed nutmeg liver, great passive congestion of lungs (two-fifths collapsed), passive congestion of spleen, and congestion of the kidneys.

#### FEEDING TESTS WITH DOGS AND MICE BY DR. HANS MOLITOR

The following additional data on the toxicity of thiocarbamide was supplied through the courtesy of Dr. Hans Molitor, Director of the Merck Institute for Therapeutic Research, Rahway, New Jersey.

#### CHRONIC TOXICITY OF THIOCARBAMIDE IN DOGS

Five young dogs from the same litter were selected for this test. Three of the dogs (males) were fed daily excepting Saturdays and Sundays with thiocarbamide, the remaining dogs (1 male and 1 female) served as controls. The thiocarbamide was administered in 10 per cent solution by stomach tube in a dose corresponding to 1 gram of substance per kilogram weight of dog, thus the total quantity of substance fed daily increased with the increasing weight of the animals.

The weight records are shown in Table III. It is evident that during the period of the test all the dogs practically doubled their weight. At no time were any toxic manifestations evidenced.

TABLE III  
INITIAL AND FINAL WEIGHTS, AND WEIGHT  
GAINS OF DOGS FED THIOCARBAMIDE

Dog No.	Dose per kg.*	Weight at start, kg.	Weight after 60 days, kg.	Total weight gain, kg.
#24 (male)	1 g.	4.61	9.10	4.49
#25 (male)	1 g.	4.70	8.90	4.20
#27 (male)	1 g.	4.70	9.40	4.70
#26 (male)	Control	6.85	13.10	6.25
#28 (female)	Control	2.60	5.00	2.40

\* Dogs were dosed five times per week.

#### TOXICITY IN MICE PER OS OF THIOCARBAMIDE

The thiocarbamide was administered per os to normal, healthy mice weighing from 15 to 25 grams. A total of 177 mice were used in these tests, not less than 15 individuals being used at each dose level.

The toxicity of thiocarbamide was as follows:

LD<sub>0</sub><sup>4</sup> — 9.0 g. per kg.

LD<sub>50</sub> — 11.5 g. per kg.

LD<sub>100</sub><sup>4</sup> — 13.0 g. per kg.

Due to the extremely low toxicity, it was necessary to use a 20 per cent solution, prepared and maintained at 37° C.

Since the osmotic pressure of a concentrated solution of thiocarbamide may exert an effect upon the toxicity, a similar experiment was conducted with a solution of sodium chloride having a like osmotic pressure, i.e., 7.6 per cent. The results, in comparison with thiocarbamide, are shown in Table IV. Since the difference in toxicity of these solutions is negligible, it can be concluded that thiocarbamide is relatively non-toxic, death being due to the influence of osmotic pressure.

TABLE IV  
TOXICITY OF ISO-OSMOTIC SOLUTIONS OF THIOCARBAMIDE  
AND SODIUM CHLORIDE FOR MICE

	20% thiocarbamide, doses in cc./kg.	7.6% sodium chloride, doses in cc./kg.
LD <sub>0</sub>	45.0	40.0
LD <sub>50</sub>	58.0	53.0
LD <sub>100</sub>	65.0	60.0

#### DR. HANS MOLITOR'S SUMMARY AND CONCLUSIONS

The chronic toxicity was run on dogs. A dose of 1 gram per kilogram body weight was fed daily except Saturdays and Sundays over a period of

<sup>4</sup> LD<sub>0</sub>=approximate values for no response; LD<sub>100</sub>=approximate values for total response.



60 days. Toward the end of the test the dogs were receiving 8 to 9 grams of substance daily. Throughout the test the dogs continued to gain weight normally and at no time showed any outward symptoms of a toxic effect of thiocarbamide. Histopathological examinations were not made.

The acute toxicity test, run on mice, was made in comparison with sodium chloride. A 20 per cent solution of thiocarbamide is of practically the same toxicity as a sodium chloride solution (7.6 per cent) of the same osmotic pressure and definitely less toxic than a 20 per cent sodium chloride solution ( $LD_{50}$ —25 cc. per kg.).

These preliminary studies on the acute and chronic toxicity of thiocarbamide indicate that it is a non-toxic substance.

#### SUMMARY

In the preliminary tests guinea pigs that were fed thiourea (thiocarbamide),  $NH_2CSNH_2$ , in amounts equivalent to approximately 45 mg. per kg. of body weight per day over a period of six weeks showed no evidence of any toxic action.

In the human feeding trial 16.8 mg. were consumed over a period of two days with no discomfort to the subject.

Rats that ingested 0.19 mg., 21.6 mg., and 49 mg. of thiourea per kg. of body weight per day for periods extending for 22 days to 28 days showed no symptoms of poisoning and made gains comparable to the controls.

On the body weight basis, the amounts of thiourea ingested by the rats referred to in the preceding paragraph are approximately 2, 250 and 570 times, and that ingested by the guinea pig in paragraph one above is 523 times, the amount consumed by a 70-kg. man in eating 200 g. of fruit treated with a 0.05 per cent thiourea solution.

The results of feeding experiments by Dr. Hans Molitor are also included, and his summary and conclusions are given in the three paragraphs preceding this summary.

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## RÔLE OF GLUTATHIONE IN THE BREAKING OF THE REST PERIOD OF BUDS BY ETHYLENE CHLOROHYDRIN

JOHN D. GUTHRIE

Potato tubers taken just after they are dug from the ground will not sprout when cut up and planted, even under the most favorable conditions. They sprout readily only after storage of about two months. From a practical standpoint it is often desirable to plant tubers for a second crop just after the first one is harvested, and for this a chemical treatment that makes the tubers sprout at once must be used. From a theoretical standpoint an understanding of the reasons for the tubers remaining dormant and the way by which they can be made to grow at once is of interest because of the relation to plant growth in general.

The chemicals commonly used for treating dormant potato tubers are ethylene chlorohydrin, thiourea, and sodium or potassium thiocyanate (2). Considerable work has been done on the chemical and physiological changes that go on in the tubers following treatment with these chemicals. The relation of many of these changes to sprouting has not been established and there is evidence that some of the changes are not the cause of sprouting (3). However, evidence has been accumulating that one of these changes, an increase in glutathione (8), may have some part in causing the tubers to sprout following treatment with ethylene chlorohydrin.

Using a newly developed method for glutathione based on its reaction with sulphur to form hydrogen sulphide (9) it was found that there was often as much as a sixfold increase in glutathione following treatment with ethylene chlorohydrin. As additional evidence, glutathione was isolated in crystalline form from potato tubers following treatment with ethylene chlorohydrin (6), while none could be isolated from untreated tubers. This isolation has since been verified by Pett (12), so that there can be no doubt that there is a large increase in glutathione following treatment with ethylene chlorohydrin.

The two other chemicals, potassium thiocyanate and thiourea, produce only small increases in glutathione content. However, according to our present ideas, these act similarly to glutathione in the dormancy breaking process, since they also contain sulphur in the divalent form. This is shown more clearly by the following diagram in which the arrow indicates the induction of glutathione formation by ethylene chlorohydrin treatments:

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Thiourea  
 $(\text{NH}_2)_2\text{CS}$

Potassium  
thiocyanate  
 $\text{KSCN}$

Ethylene  
chlorohydrin  
 $\text{CH}_2\text{Cl CH}_2\text{OH}$

↓

GSH

Glutathione

Stated in another way, ethylene chlorohydrin breaks the rest period indirectly by increasing the glutathione content, while potassium thiocyanate and thiourea act more directly and substitute for glutathione. This offers an explanation for such widely different chemicals as ethylene chlorohydrin and potassium thiocyanate having a similar effect on the rest period.

With the above idea in mind it was natural to try the direct effect of glutathione on freshly-harvested potato tubers, and compare the sprouting response with that of ethylene chlorohydrin treatments inducing a similar glutathione content in the tissue. This was rendered difficult by the slowness with which glutathione entered the tissue, making it necessary to use high concentrations of glutathione. For reasons of economy most of the glutathione used was isolated from yeast. About 15 g. can be isolated from 20 kg. and some can be recovered from the used solutions at the conclusion of the treatments.

Since ethylene chlorohydrin also breaks the rest period of buds of woody plants as shown by Denny and Stanton (4), the effect of glutathione treatments on the resting buds of pear and peach was also investigated.

#### EXPERIMENTS WITH POTATO TUBERS

*Glutathione treatments.* Approximately cubical pieces of freshly-harvested potato tubers (*Solanum tuberosum* L.) of the Irish Cobbler variety were cut with the bud in the center of one side. These pieces weighed about 10 g. They were washed after cutting, dried with cheesecloth and placed in Petri dishes with the bud side up, except in one experiment in which the pieces were cut with the buds at the basal side of the pieces and the pieces placed in the dish with the apical side down. After arranging the pieces in Petri dishes, enough glutathione solution was poured into the dish to come about two-thirds of the way up the sides of the pieces. Control pieces were similarly treated with water. The glutathione concentrations tried ranged from 1 to 4 g. per 100 cc. and the time of treatment from two to six days. With the six-day treatments a thin slice was cut from the base of each piece after three days, and the old solution replaced with a fresh one. All of the glutathione treatments were made at 10° C. At the conclusion of the treatment period, the pieces were planted in soil.

*Ethylene chlorohydrin treatments.* These treatments were made according to the method of Denny (2). About one-fourth to one-twelfth of the recommended amount of ethylene chlorohydrin was used and the duration of



treatment was shorter than ordinarily used for potato tubers. This was necessary because the recommended amount of ethylene chlorohydrin induced a larger increase in the glutathione content of the tissue than could be obtained by the methods available at present for direct treatment with glutathione. The object of the experiments was to observe the sprouting response of pieces of like glutathione content obtained by (a) treatment with ethylene chlorohydrin, and (b) treatment with solutions of glutathione. Whole tubers were placed in glazed earthenware crocks so as to half fill the container. About a kilogram of tubers was required. From 0.2 to 0.6 cc. of 40 per cent ethylene chlorohydrin were then measured on cotton and placed above the tubers. The crocks were then covered and sealed with modeling clay. After 24 hours at about 25° C. the tubers were cut into approximately cubical 10-g. pieces with the eye in the center and planted in soil.

*Glutathione determinations.* Some of the pieces were used for glutathione determinations one week after the beginning of treatment, with the exception of the six-day glutathione treatments that were sampled nine days after the beginning of treatment. The pieces were washed, dried with cheesecloth, and the callus tissue cut off in a thin layer. Usually six pieces were used for the glutathione determinations, and a 25-g. sample and one-half quantities of reagents used. With the ethylene chlorohydrin treatments more pieces were available, so 12 pieces were usually sampled and 50-g. samples used. The glutathione was determined by a method based on its reaction with sulphur to form hydrogen sulphide. In experiments 1 and 2 a procedure involving an empirical correction was used, but in experiments 3 and 4 an improved procedure (9) was followed.

*Sprouting response.* The effect on the sprouting was obtained in the usual manner. The pieces were planted in flats in lots of 12 and placed outside or in the greenhouse depending on the season. One flat of 12 pieces was used for each of the glutathione treatments and duplicate flats of 12 pieces for each of the ethylene chlorohydrin treatments. The number of sprouts above ground was recorded twice a week and the time in days for one-half of the pieces to show sprouts above ground. This value is essentially the time required for the median piece to sprout.

*Results.* The results of four experiments carried out as described above are summarized in Table I. Experiment 1 is inconclusive, but by no means inconsistent with the results of the other experiments. The rather weak glutathione treatments had little effect on the sprouting, but an ethylene chlorohydrin treatment that produced a larger increase in glutathione also had little effect on the sprouting. Experiment 2 is quite conclusive, however, since a marked increase in sprouting was obtained and the glutathione content of the glutathione treated lot and of the ethylene chlorohydrin treated lot was about the same. The sprouting response was also about the same as would be expected if glutathione played a part in the breaking of

the rest period by ethylene chlorohydrin. This is also shown in Figure 1 in which the number of sprouts above ground is plotted against days from the time of planting. It will be seen that the curves for the glutathione treated lots and for the ethylene chlorohydrin treated lots are very similar. In experiment 3 the treatments with glutathione shortened the sprouting time

TABLE I

A COMPARISON OF THE SPROUTING RESPONSE OF FRESHLY-HARVESTED POTATO TUBERS AFTER GLUTATHIONE TREATMENT WITH THE SPROUTING RESPONSE FOLLOWING ETHYLENE CHLOROHYDRIN TREATMENTS THAT INDUCED A SIMILAR GLUTATHIONE INCREASE

Exp. No.	Treatment*	GSH content mg. per 100 g. tissue	Days for 1/2 above ground
1	Glutathione, 1 g. per 100 cc. 2 day basal soak	29.0	50
	Control, H <sub>2</sub> O 2 day basal soak	11.3	54
	Ethylene chlorohydrin, 0.6 cc. 40 per cent per kg.	48.2	17
	Ethylene chlorohydrin, 0.3 cc. 40 per cent per kg.	38.2	82
	Control, closed container only	14.7	92
2	Glutathione, 2 g. per 100 cc. 2 day basal soak	31.6	50
	Control, H <sub>2</sub> O 2 day basal soak	8.6	88
	Ethylene chlorohydrin, 0.2 cc. 40 per cent per kg.	35.0	50
3	Control, closed container only	13.0	83
	Glutathione, 2 g. per 100 cc. 3 day basal soak	12.8	29
	Glutathione, 4 g. per 100 cc. 2 day basal soak	18.3	32
	Control, H <sub>2</sub> O 3 day basal soak	5.6	80
	Ethylene chlorohydrin, 0.4 cc. 40 per cent per kg.	31.9	32
4	Ethylene chlorohydrin, 0.2 cc. 40 per cent per kg.	19.2	45
	Control, closed container only	8.9	55
	Glutathione, 2 g. per 100 cc. 6 day basal soak	44.5	26
	Control, H <sub>2</sub> O 6 day basal soak	6.2	84
	Glutathione, 2 g. per 100 cc. 6 day basal soak**	37.4	35
	Control, H <sub>2</sub> O 6 day basal soak**	6.9	94
	Ethylene chlorohydrin, 0.4 cc. 40 per cent per kg.	35.5	45
	Control, closed container only	8.2	73

\* The amounts of ethylene chlorohydrin used were much lower than recommended for production of optimal sprouting of freshly-harvested potato tubers, since the purpose was to induce glutathione formation in amounts comparable to that found in tubers treated with glutathione itself.

\*\* Bud at basal side of piece, apical end in solution.

considerably and appear to be more effective than an ethylene chlorohydrin treatment producing about the same increase in glutathione. In experiment 4 the glutathione treatments produced rather large increases in the glutathione content of the tissue and also shortened the sprouting time. The ethylene chlorohydrin treatment which produced about the same glu-

tathione increase, shortened the sprouting time almost as much as the direct treatment with glutathione. A photograph taken after 58 days of some of the treatments of experiment 4 is shown in Figure 2 A. The pieces in the flat on the left were treated with a solution of glutathione, those in the center with water, and those on the right with ethylene chlorohydrin.

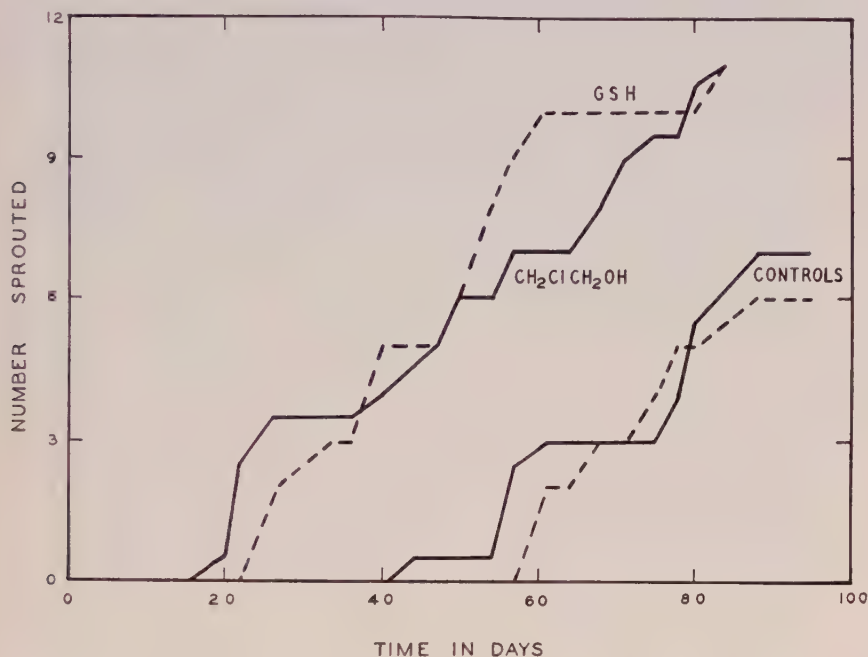


FIGURE 1. A comparison of the effect of glutathione treatment on the sprouting of freshly-harvested potato tubers with an ethylene chlorohydrin treatment that induced about the same glutathione content in the tissue. Broken lines are glutathione treatment and corresponding control. Solid lines are ethylene chlorohydrin ( $\text{CH}_2\text{ClCH}_2\text{OH}$ ) treatment and corresponding control.

The above experiments show that treatment of freshly-harvested potato tubers with glutathione hastens their sprouting. Also, if allowance is made for experimental difficulties and natural variation of the tubers, the experiments offer evidence that for a given increase in glutathione, the effect on sprouting is essentially the same whether this increase is brought about directly by treatment with glutathione itself, or indirectly by treatment with ethylene chlorohydrin.

#### EXPERIMENTS WITH THE RESTING BUDS OF PEAR AND PEACH

The foregoing experiments with potato tubers in which glutathione treatments shortened the rest period suggested that glutathione might be



FIGURE 2. (A) Effect of glutathione and ethylene chlorohydrin on the sprouting of freshly-harvested potato tubers. Pieces in flat on left treated with glutathione, pieces in flat in center treated with water, pieces in flat on right treated with ethylene chlorohydrin. Glutathione content of tissue 37.4, 6.9, and 35.5 mg. per 100 g. respectively. (B) Effect of glutathione treatments on the growth of buds of pear. The ten branches on left were treated with glutathione and the ten on the right with water. (C) Effect of glutathione on the growth of buds of peach. Tagged twig on left treated with glutathione, tagged twig on right with water, other twigs untreated.



effective with other plants. Interest in pear and peach buds was brought about by a paper by Bennett and Skoog (1) in which they reported that injection of yeast extracts into the ends of branches of trees during the rest period induced the buds to grow. Since yeast is one of the richest sources of glutathione, it was thought likely, in view of the results with potato tubers, that it was glutathione in the yeast extracts that produced the stimulating effect on the growth of the buds. Furthermore, Denny and Stanton (4) have shown that ethylene chlorohydrin will break the rest period of buds of woody plants. For these reasons glutathione was tried on the buds of peach and pear and proved to be effective in inducing growth of the resting buds.

The solutions used were injected by cutting off the end of the twig, attaching an empty drying tube with rubber tubing to the cut end, placing the solution in the tube and allowing it to be drawn into the tree. Professor J. P. Bennett of the University of California kindly furnished the details of this method of injection in a personal communication.

The first two experiments with pear buds (*Pyrus communis* L.) were started the first week in September. Potted trees of the Bartlett variety, which had grown the previous winter in the greenhouse and placed out-of-doors in the spring, were used. They had ceased growth, shed most of their leaves, and entered the rest period early in the summer. In experiments 1 and 2, each of ten branches were injected with 50 mg. of glutathione in 10 cc. of water. Ten branches injected with water served as controls. The solution was taken up readily in most cases, but a few branches did not take up all of the solution. The number of buds growing on each of the branches was recorded after 28 days. In a few cases, injury to the terminal bud due to the high local concentration of glutathione was observed. The results are summarized in Table II. A statistical examination by the method given by Fisher (5, p. 107) of the values for the individual branches showed odds of over 1000 to 1 in both experiments that the glutathione treatments had induced more buds to grow than had the water controls. The branches from experiment 1 were cut off after two months and photographed as shown in Figure 2 B.

TABLE II  
EFFECT OF INJECTION OF GLUTATHIONE ON THE RESTING BUDS OF PEAR

Exp. No.	Number buds growing per 10 branches	
	GSH	H <sub>2</sub> O
1	100	27
2	69	18
3	49 71 59 72	19

In experiment 3, potted trees that had grown out-of-doors during the summer were removed to the greenhouse in November. Four samples of glutathione of various degrees of purity were tried to see if an impurity in the glutathione could be causing the effect on the buds. This did not prove to be the case, since the response was essentially the same with all four samples. The samples of glutathione used in experiment 3 in the same order as the tabulation of data in the table were: (1) preparation from yeast purified by a second precipitation with  $\text{Cu}_2\text{O}$  and two recrystallizations; (2) preparation from yeast, not specially purified, but traces of copper removed with kaolin; (3) sample from Eastman Kodak Company; (4) sample from Hoffman-LaRoche and Company.

In the experiment with peach buds (*Prunus persica* Sieb. & Zucc.) ten twigs were injected with a solution of glutathione, 25 mg. in 5 cc. of water being used for each branch. Ten similar twigs were injected with water. These twigs were on potted trees of the Elberta variety that had been taken into the greenhouse about November 15. The treatments were made December 7. Fifteen days after the beginning of treatment, 16 buds were growing on six of the twigs injected with glutathione, while no buds were growing on the control twigs. After 35 days from the beginning of the experiment, 17 buds were growing on six of the twigs injected with glutathione, while two buds were growing on two of the control twigs. This shows that injection of peach twigs during the rest period with a solution of glutathione induces the buds to grow. This is further illustrated by Figure 2 C in which twigs typical of the above experiment are shown after 36 days.

#### DISCUSSION

The above results support the idea that glutathione plays an important part in the breaking of the rest period by ethylene chlorohydrin. The essential facts in evidence are that ethylene chlorohydrin induces a large increase in the glutathione content of potato tissue and that glutathione itself will break the rest period of buds. It would appear also that the effect of glutathione on the rest period of potato tubers is about the same as brought about by ethylene chlorohydrin treatments that induce a similar glutathione content in the tissue. The data, however, are not sufficiently extensive to permit decision of the question of whether glutathione is the only factor involved in the breaking of the rest period by ethylene chlorohydrin. In fact it would be unreasonable to assume that the many changes that ethylene chlorohydrin treatments induce in the tissue have no effect on the sprouting response.

Although glutathione is the only factor at present for which sufficient evidence is available to indicate that it plays a part in the breaking of the rest period by ethylene chlorohydrin, it should be pointed out that it is not

known how glutathione breaks the rest period. Neither is it known how potassium thiocyanate and thiourea break the rest period. These compounds do not induce much of a glutathione increase in the tissue, but they do contain divalent sulphur, as does glutathione. Furthermore, Miller (11) has shown that a number of other compounds containing divalent sulphur are very effective in breaking the rest period. The idea expounded by Hammett (10) that compounds containing reduced sulphur, especially sulphydryl compounds, play an essential part in the process of cell division may be also cited on this point.

Some non-sulphur compounds such as ethyl alcohol and acetaldehyde which are somewhat effective in breaking the rest period also increase the glutathione content of potato tubers (7), although results with these leave much to be desired from a quantitative standpoint, since they appear to be somewhat more effective than might be predicted from their effect on the glutathione content of the tissue. Another point in evidence is that ethylene chlorohydrin breaks the rest period and increases the sulphydryl content and presumably the glutathione content of gladiolus corms and Jerusalem artichoke tubers. However, probably because of the difficulties involved in work with woody tissues, it has not yet been possible to show a glutathione increase with pear or peach although ethylene chlorohydrin will break their rest period and glutathione is also effective as shown in the present paper.

The glutathione content does not change much when potato tubers slowly come out of the rest period during storage, but this is not, as might appear on first consideration, evidence against the idea that glutathione plays a part in the breaking of the rest period. The tubers do contain some glutathione, and this small amount of glutathione acting over a long period could have ultimately the same effect as a larger amount acting over a shorter period. This would be especially true if glutathione acted to hasten some chemical change more directly connected with the breaking of the rest period than glutathione itself.

It appears that ethylene chlorohydrin induces an increase in the glutathione content of the tissue and that the glutathione acts in some way to break the rest period, perhaps by speeding up some process intimately associated with the rest period. Certain compounds containing divalent sulphur act similarly to glutathione and substitute for it in the process of breaking the rest period. This concept somewhat simplifies the problem of how certain substances break the rest period, since the question is not how such chemically diverse substances as ethylene chlorohydrin on one hand and potassium thiocyanate and thiourea on the other break the rest period, but how certain compounds that contain divalent sulphur, such as glutathione, potassium thiocyanate, and thiourea break the rest period.

## SUMMARY

Treatment of freshly-harvested potato tubers with solutions of glutathione shortens the rest period. The effect on the rest period is about the same as that of ethylene chlorohydrin treatments that induce a similar glutathione content in the tissue. Injection of solutions of glutathione into the branches of pear and peach shortens the rest period of the buds. These results indicate that ethylene chlorohydrin breaks the rest period by increasing glutathione in the tissue.

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FORMATION OF  $\beta$ -*o*-CHLOROPHENYL-GENTIOBIOSIDE  
IN GLADIOLUS CORMS FROM ABSORBED  
*o*-CHLOROPHENOL

LAWRENCE P. MILLER

Investigations in connection with the action of ethylene chlorohydrin in breaking the rest period of potato tubers (*Solanum tuberosum* L.) and *Gladiolus* corms (4, 5, 6) have shown that both of these tissues convert the absorbed ethylene chlorohydrin into  $\beta$ -2-chloroethyl-*d*-glucoside (11, 13). In extending these studies to other plant tissues and to additional chemicals it has been found that the formation of  $\beta$ -glycosides from absorbed non-naturally occurring substances can take place quite generally among the higher plants (12). The sugar component of such glycosides is, however, not always *d*-glucose, just as in the case of the natural glycosides. Thus the glycoside formed by *gladiolus* corms from *o*-chlorophenol is not  $\beta$ -*o*-chlorophenyl-*d*-glucoside (14) even though corms of the same variety form a  $\beta$ -glucoside when ethylene chlorohydrin is the aglucon furnished.

The *o*-chlorophenyl glycoside from *gladiolus* corms was obtained in crystalline form as the acetyl derivative. The chlorine content of the acetylated glycoside corresponds to that required for the heptaacetate of an *o*-chlorophenyl glycoside involving a C<sub>12</sub> disaccharide. Tests with partially purified preparations of the glycoside from aqueous extracts of the corms showed that hydrolysis by emulsin results in the release of two molecules of reducing sugar, calculated as glucose, for each molecule of *o*-chlorophenol set free. The linkage between the two sugar molecules of the disaccharide is therefore also a  $\beta$  linkage. Preliminary studies on the identification of the sugars by the benzimidazole procedure (16)<sup>1</sup> indicated that the disaccharide consists of two molecules of glucose. These results suggested that the glycoside might be a gentiobioside and accordingly  $\beta$ -*o*-chlorophenyl-gentiobioside heptaacetate<sup>2</sup> was synthesized (7, 17) and found to be identical with the acetylated glycoside from *gladiolus* corms. The corresponding propionyl derivatives were also prepared and found to be identical. The glycoside produced by *gladiolus* corms is therefore shown to be  $\beta$ -*o*-chlorophenyl-gentiobioside. These results have been discussed briefly in a previous preliminary note (15).

<sup>1</sup> The author is indebted to Drs. Stanford Moore and Karl Paul Link for providing him with a copy of this paper prior to its publication.

<sup>2</sup> The  $\beta$ -octaacetyl gentiobiose used in this synthesis was kindly supplied by Prof. William Lloyd Evans of Ohio State University.

## EXPERIMENTAL

TREATMENT OF GLADIOLUS CORMS WITH *o*-CHLOROPHENOL

The properties of *o*-chlorophenol are such as to make this substance very suitable for investigations on glycoside formation in plant tissue from introduced aglucons. It is sufficiently volatile so that if an aqueous solution is concentrated to half its volume, the *o*-chlorophenol is substantially all found in the distillate in which it can readily be determined by adding an excess of bromine in the form of a 0.1 N potassium bromate solution and determining the excess bromine iodometrically under the conditions used for the quantitative determination of phenol (1, p. 281). Four atoms of bromine are absorbed for every molecule of *o*-chlorophenol present. It is therefore possible to test crude plant extracts for the presence of an *o*-chlorophenyl glycoside by hydrolyzing with emulsin and determining the amount of *o*-chlorophenol set free. This is obviously more satisfactory than a determination of the increase in reducing sugars after emulsin hydrolysis since such increases could arise from natural glycosides present in the tissue extract, and possibly also from the hydrolysis of other carbohydrates since emulsin is a mixture of enzymes. The non-ionic chlorine in the *o*-chlorophenol also serves as a "tag" by which the introduced chemical can be followed since the higher plants do not naturally contain non-ionic chlorine.

The gladiolus corms were treated with *o*-chlorophenol by placing 1500 to 2000 g. in a desiccator with a capacity of about seven liters and drawing a stream of air, which had first passed through two Van Slyke-Cullen (18) tubes containing *o*-chlorophenol, through the desiccator. After continuing the exposure for 72 hours in this way the tubes containing the chemical were removed from the system and air drawn through for another seven to ten days. The corms were then ground through a meat chopper, using the fine cutter, and the juice expressed by squeezing through cheesecloth. The expressed juice was centrifuged to remove starch and other suspended matter, heated to 80° C. to inactivate enzymes and remove substances which precipitate on heating, and again centrifuged. A further extract was obtained by covering the residues with water, mixing thoroughly, and again squeezing through cheesecloth. This extract was also heated to 80° and centrifuged.

In order to determine the amount of *o*-chlorophenyl glycoside present a portion of the juice was subjected to the action of emulsin in a M/20 acetate buffer of pH 4.75, containing 50 mg. of emulsin per 100 cc., at 35° for 18 to 24 hours. The amount of *o*-chlorophenol released was then determined by concentrating these solutions to half their original volume and titrating the distillates as indicated above. The values obtained with a blank determination containing no emulsin were a measure of the free *o*-chlorophenol remaining together with any that may have been set free dur-

TABLE I  
FORMATION OF A GLYCOSIDE HYDROLYZABLE BY EMULSIN BY GLADIOLUS  
CORMS TREATED WITH *o*-CHLOROPHENOL

Variety	Millimols <i>o</i> -chlorophenol recovered from 100 cc. expressed juice	
	After emulsin hydrolysis	Without emulsin hydrolysis
Mixed	0.69	0.10
"	1.48	0.04
"	0.95	0.03
"	0.90	0.03
"	0.83	0.05
"	1.45	0.05
"	0.98	0.08
Giant Nymph	1.50	0.18
Alice Tiplady	0.60	0.00

ing the process of extraction. The results with treatments of nine lots of gladiolus corms are shown in Table I. It is seen that the quantities of *o*-chlorophenol recovered without previous emulsin hydrolysis are quite small showing that little of the absorbed chemical remained unchanged at the time of sampling. The amount of *o*-chlorophenyl glycoside, calculated as  $\beta$ -*o*-chlorophenyl-gentiobioside, found in the expressed juice averaged about 0.5 gram per 100 cc. Since the hydrolysis by emulsin is probably not 100 per cent complete, the quantities present were most likely a little higher than the figures in the table indicate.

*Isolation of  $\beta$ -o-Chlorophenyl-Gentiobioside as the Heptaacetate from Treated Corms*

After numerous attempts to obtain the glycoside formed in the gladiolus corms from the absorbed *o*-chlorophenol in crystalline condition, the following rather simple procedure for obtaining it as the acetyl derivative was developed. Aqueous extracts of the treated corms were precipitated with an excess of lead acetate, filtered, and the excess lead removed by precipitation with  $H_2S$ . The  $H_2S$  remaining in the filtrate after filtering off the lead sulphide was driven off by passing a current of nitrogen through the solution. The solution was then concentrated under diminished pressure to a volume suitable for transfer to a Kutscher and Stendel type of continuous extractor with a capacity of 500 cc. The material was extracted with ethyl acetate over a period of a number of days since the glycoside is taken up quite slowly by the ethyl acetate. It is necessary to change the flask containing the ethyl acetate several times during this prolonged extraction period since the materials taken out settle to the bottom of the flask as a white amorphous deposit and if heated too long, some darkening occurs. The combined ethyl acetate solutions were washed with water three or four times and these aqueous solutions evaporated to dryness

under diminished pressure. Several portions of absolute ethanol were added toward the end of the evaporation to aid in driving off the last traces of moisture. The residue was then acetylated by adding acetic anhydride and pyridine and allowing the mixture to stand overnight at room temperature. The reaction mixture was then poured, slowly, with constant stirring into five times its volume of ice water. After several hours the solution was filtered and the precipitate washed with cold water. The precipitate was dissolved in a mixture of acetone and absolute ethanol, heated with Norite, and filtered. After standing for some time at room temperature, during which some of the acetone evaporates, the acetylated glycoside crystallizes out.

The extraction with ethyl acetate in the continuous extractor removes fatty materials and other substances in addition to the glycoside. These impurities are extracted more rapidly than the glycoside so that after the extraction has proceeded for some time subsequent extracts are colorless and the extracts can be acetylated directly after removal of the ethyl acetate by evaporation. The crude product obtained on pouring into water and filtering is crystalline and may have a melting point only a degree or two lower than the purified recrystallized product. The acetylated glycoside will also crystallize quite readily, however, from very impure mixtures, the other products of the acetylation remaining behind as oily non-crystallizable materials.

When aqueous extracts of *gladiolus* corms which had not been treated with *o*-chlorophenol were carried through the above procedure, no crystalline product was obtained.

The acetylated *o*-chlorophenyl glycoside was first obtained from the earlier treatments in which the variety of *gladiolus* used was either unknown or the corms used consisted of several varieties which had become mixed. The experiments were then repeated with the Giant Nymph variety and with the Alice Tiplady variety. The latter variety was included because it had been the one used for the experiments with ethylene chlorohydrin in which it had been shown that  $\beta$ -2-chloroethyl-*d*-glucoside was formed (11). The crystalline acetylated glycoside obtained after treatment with *o*-chlorophenol was the same for all the varieties included in this study, thus showing that the same variety may form a glycoside involving different sugars depending upon the aglucon. A total of about 8 grams of the acetylated *o*-chlorophenyl glycoside was prepared in these investigations. After several recrystallizations from absolute ethyl alcohol it melted at 207.5–208.5° (corr.) and had a specific rotation  $[\alpha]_D^{25} = -49.4^\circ$  (CHCl<sub>3</sub>, concn., 2.66 g. in 100 cc.). A mixed melting point determination with synthetic  $\beta$ -*o*-chlorophenyl-gentiobioside heptaacetate showed no depression.



*Analysis:*<sup>3</sup> Theory for  $\beta$ -o-chlorophenyl-gentiobioside heptaacetate,  $C_{32}H_{39}O_{18}Cl$ : C, 51.44; H, 5.26; Cl, 4.75. Found: C, 51.46, 51.63; H, 5.34, 5.09; Cl, 4.98, 4.63.

*Deacetylation of the Isolated  $\beta$ -o-Chlorophenyl-Gentiobioside Heptaacetate*

Three grams of the isolated  $\beta$ -o-chlorophenyl-gentiobioside heptaacetate were suspended in 75 cc. of absolute methyl alcohol and the solution cooled in an ice-salt bath. The equivalent of 5 cc. of 0.5 N barium methylate solution was added and the mixture kept at 5° C. for 20 hours (8). The barium methylate was then neutralized by adding the exact equivalent of 0.5 N  $H_2SO_4$  and the solution filtered through Celite and Norite and evaporated to dryness under diminished pressure. The residue was dissolved in hot absolute alcohol and on cooling the glycoside separated out in the form of a gelatinous precipitate containing much occluded solvent. Attempts to obtain the glycoside in the crystalline state with the use of various solvents and conditions of crystallization have as yet not been successful. On drying the gelatinous precipitate obtained as indicated above, the glycoside remains as a white amorphous residue.

*Hydrolysis by emulsin.* Some of this amorphous glycoside was used to repeat previous experiments made on less pure material to determine the amount of reducing sugar and o-chlorophenol liberated by emulsin hydrolysis. Thus, in one test 53.2 mg. were subjected to the action of emulsin at pH 4.75 for 22 hours at 35° C. In a control sample the solution with the emulsin was heated to boiling before the addition of the glycoside. At the end of the period of hydrolysis a portion of the samples was distilled for the recovery of the o-chlorophenol set free and another portion was analyzed for the sugar content. Emulsin hydrolysis resulted in freeing 0.097 millimol of o-chlorophenol and 0.192 millimol of reducing sugar calculated as glucose.

*Preparation of the heptapropionate.* About 110 mg. of the amorphous glycoside were dissolved in 5 cc. dry pyridine and 3 cc. propionic anhydride added. The reaction was allowed to proceed overnight at room temperature after which the mixture was poured into 75 cc. of ice water. After standing for several hours the crystalline product was filtered off and washed with cold water. Without drying, the product was recrystallized from a mixture of acetone and absolute alcohol. Yield 0.19 g., melting point 176.5–177.5°. After two more recrystallizations from 95 per cent ethanol 0.15 g. melting at 178.5–179° was obtained. No depression resulted in a mixed melting point determination with synthetic  $\beta$ -o-chlorophenyl-gentiobioside heptapropionate. The specific rotation was  $[\alpha]_D^{26} = -38.0^\circ$  ( $CHCl_3$  concn., 2.44 g. per 100 cc.).

<sup>3</sup> Microanalyses by H. Jeanne Thompson.

*Analysis:*<sup>3</sup> Theory for  $\beta$ -*o*-chlorophenyl-gentiobioside heptapropionate,  $C_{39}H_{53}O_{18}Cl$ : Cl, 4.19. Found: Cl, 4.40, 4.20.

SYNTHESIS OF  $\beta$ -*o*-CHLOROPHENYL-GENTIOBIOSIDE  
HEPTAACETATE AND HEPTAPROPIONATE

*Synthesis of  $\beta$ -*o*-Chlorophenyl-Gentiobioside Heptaacetate*

$\beta$ -*o*-Chlorophenyl-gentiobioside heptaacetate was synthesized by a procedure based on that used by Helferich and Schmitz-Hillebrecht (7) for the preparation of  $\beta$ -phenyl-gentiobioside heptaacetate. A mixture of 4 g. of  $\beta$ -octaacetyl gentiobiose<sup>2</sup>, 5.8 g. of *o*-chlorophenol and 0.06 g. of *p*-toluenesulphonic acid was heated in an oil bath for ten minutes at 110°, followed by 20 minutes during which the temperature was increased to 120°, which was maintained for another ten minutes. On cooling the melt was taken up in 40 cc. of benzene and the benzene solution washed four times with water. The benzene solution was then evaporated under reduced pressure and the evaporation continued with the addition of several portions of water which aided in removing the excess *o*-chlorophenol. During the course of the evaporation the product separated out in crystalline form and three crops totaling 0.775 gram, melting at about 203.5°, were obtained. This represents a yield of 17.6 per cent of crude product. After repeated recrystallization from absolute alcohol and finally from a mixture of absolute alcohol and ethyl acetate the product melted at 207.5–208.5° and had a specific rotation of  $[\alpha]_D^{24} = -49.7^\circ$  ( $CHCl_3$ , concn., 2.545 g. in 100 cc.). As stated above no depression of melting point resulted in a mixed melting point determination with the isolated acetylated *o*-chlorophenyl glycoside from gladiolus corms.

*Analysis:*<sup>3</sup> Theory for  $C_{32}H_{39}O_{18}Cl$ : C, 51.44; H, 5.26; Cl, 4.75. Found: C, 51.55, 51.36; H, 5.00, 4.88; Cl, 4.50, 4.69.

*X-ray diffraction pattern of synthetic and isolated glycosides.* Through the courtesy of Dr. Wayne A. Sisson the X-ray diffraction patterns of synthetic  $\beta$ -*o*-chlorophenyl-gentiobioside heptaacetate and the isolated acetylated glycoside from gladiolus corms were obtained and are shown in Figure 1. These patterns are seen to be identical and thus offer further evidence that the glycoside formed in gladiolus corms is the  $\beta$ -gentiobioside.

*Preparation of Synthetic  $\beta$ -*o*-Chlorophenyl-Gentiobioside  
Heptapropionate*

Synthetic  $\beta$ -*o*-chlorophenyl-gentiobioside heptaacetate (0.35 g.) was dissolved in 15 cc. dry methanol and the solution cooled in an ice-salt bath (8). The equivalent of 2 cc. of 0.5 N barium methylate was added and the mixture kept at 5° overnight. The barium methylate was then neutralized with 0.5 N  $H_2SO_4$ , the solution filtered through Celite and Norite and the filtrate evaporated to dryness *in vacuo*. The residue was dissolved in 10 cc.

dry pyridine and 5 cc. propionic anhydride added. After standing at room temperature overnight the mixture was poured into 100 cc. of ice-water and the crystalline product separated by filtration. The crude product was dissolved without previous drying in acetone and set aside for crystallization after the addition of an equal volume of absolute ethyl alco-

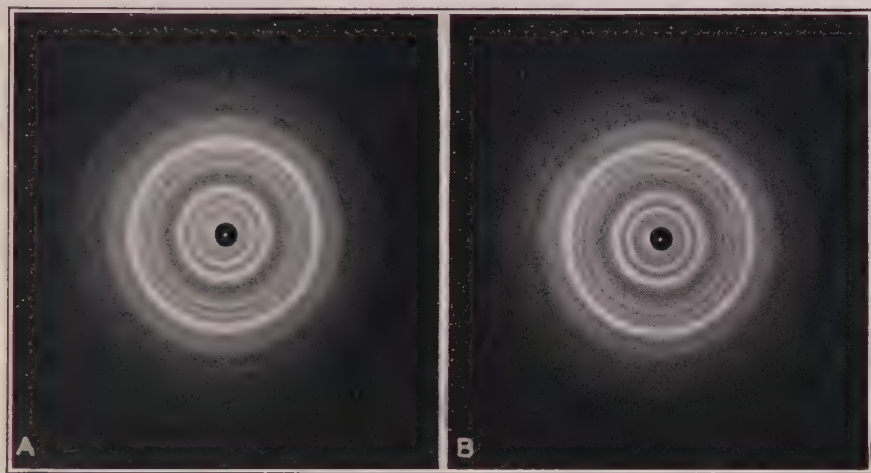


FIGURE 1. X-ray diffraction patterns of A, acetyl derivative of *o*-chlorophenyl glycoside from gladiolus corms; B, synthetic  $\beta$ -*o*-chlorophenyl-gentiobioside heptaacetate.

hol. Yield, 0.38 g. It was recrystallized once from 95 per cent ethanol. Melting point, 178.5–179.0°,  $[\alpha]_D^{26} = -38.0^\circ$  ( $\text{CHCl}_3$  concn., 2.605 g. in 100 cc.).

*Analysis*:<sup>3</sup> Calculated for  $\text{C}_{39}\text{H}_{53}\text{O}_{18}\text{Cl}$ : Cl, 4.19. Found: Cl, 4.33, 4.25.

#### DISCUSSION

Gentiobiose is not known to be widely distributed among plants. As the sugar component of the glycoside amygdalin it is found in many species of Rosaceae and as a constituent of the trisaccharide gentianose it is found in the rhizomes of Gentianaceae (19, p. 863). It has also been identified as the sugar component of  $\alpha$ -crocin (9) and has been shown to be present in an extract of *Poa trivialis* (3). Whether gentiobiose is present in appreciable quantities in untreated gladiolus corms is not clear; however, when expressed juice of untreated corms is subjected to the action of emulsin, the increase in reducing sugars is quite small (10, p. 217), which would indicate that only small amounts are present. It is quite likely, therefore, that the treatments with *o*-chlorophenol induce the formation of much or probably most, or perhaps even all, of the gentiobiose which enters into glycoside formation. That treatment with a chemical may markedly alter the chemi-

cal composition of plant tissue has been amply shown in the case of ethylene chlorohydrin in numerous papers from this Institute. It has also been found that gentiobiosides are formed in tomato plants from absorbed chemicals (15) and it would thus appear that gentiobiose may be more widely distributed among plants than was previously supposed.

Since many different types of natural glycosides are found in plants, it is impossible to ascribe a role to plant glycosides in general. It has long been thought, however, that glycoside formation in plants may serve as a means of putting harmful waste products out of action (2, p. 98). These experiments with non-naturally occurring substances offer evidence that detoxication may be one role of glycoside formation in plants, if it is true, which seems likely, that the resulting glycosides are less harmful to the plant than the more reactive aglucons. It is of interest that these induced glycosides do not move readily in the plant. Unpublished experiments with ethylene chlorohydrin treated potato tubers and gladiolus corms have shown that when such tubers or corms, which contain  $\beta$ -2-chloroethyl-*d*-glucoside, are planted the resulting sprouts do not contain any of the glucoside. Similarly when gladiolus corms containing *o*-chlorophenyl-gentiobioside were grown until new corms were produced, the resulting top and new corms did not contain a  $\beta$ -*o*-chlorophenyl-glycoside, although appreciable quantities were still present in the old corms. It would thus appear to be literally true that these introduced chemicals are put out of action through glycoside formation.

#### SUMMARY

When gladiolus corms are treated with *o*-chlorophenol, the absorbed *o*-chlorophenol is converted into a  $\beta$ -glycoside. Through the synthesis of  $\beta$ -*o*-chlorophenyl-gentiobioside heptaacetate and heptapropionate, which were found to be identical with the corresponding derivatives of the glycoside formed in the treated corms, this glycoside has been shown to be  $\beta$ -*o*-chlorophenyl-gentiobioside.

Since gladiolus corms had previously been found to form  $\beta$ -2-chloroethyl-*d*-glucoside from absorbed ethylene chlorohydrin, the results show that in the same plant tissue the sugar component of glycosides arising as a result of the absorption of chemicals which serve as aglucons may differ with different chemicals.

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# STUDIES ON THE DEVELOPING COTTON FIBER. II. IDENTIFICATION AND ESTIMATION OF THE REDUCING SUGARS<sup>1</sup>

JACK COMPTON AND FORREST E. HAVER, JR.

The investigations of Ivanova and Kurenova (15) and of Gallup (10) on the constituents of the developing cotton fiber included determinations of the total reducing sugars. The assumption was made in each case, however, that the reducing sugars were composed entirely of glucose. In recognition of the variety of substances occurring in the cell wall of the cotton fiber the validity of this assumption is questionable. The present study is therefore directed toward the determination and quantitative estimation of the various reducing sugars present in developing cotton fibers. To determine the effect of environmental conditions on the reducing sugars in the fibers, the cotton plants were grown under both field and greenhouse conditions.

## EXPERIMENTAL

### MATERIALS

The cotton bolls used in this study were obtained from cotton plants, Super Seven variety (*Gossypium hirsutum* L. Strain 4), grown under field and greenhouse conditions. The methods of selecting the cotton bolls have been described in a previous publication (7). In contrast to these methods in which the cotton bolls were selected from different cotton plants, data were obtained concerning the weights of the wet and dry crude cotton fibers separated from bolls at various ages when selected from a single healthy cotton plant grown in the greenhouse (Table I).

TABLE I

AVERAGE WEIGHTS AND PERCENTAGES OF THE FIBROUS CONSTITUENT OF COTTON BOLLS  
OBTAINED FROM A SINGLE COTTON PLANT GROWN UNDER GREENHOUSE CONDITIONS

Days after flowering	Wt. of boll, g.	Fiber + H <sub>2</sub> O + waxes + sugars		Dry fiber after H <sub>2</sub> O extraction		
		Wt., g.	% boll wt.	Wt., g.	% boll wt.	% wet fiber wt.
10	6.89	3.14	45.5	0.039	0.57	1.24
15	13.41	3.41	25.4	0.171	1.28	5.01
20	21.64	5.77	26.6	0.280	1.33	5.00
25	17.97	4.63	25.7	0.308	1.71	6.65
30	22.54	5.89	26.1	0.589	2.61	10.00
35	23.12	6.68	28.8	1.077	4.66	16.12
40	25.76	6.98	27.1	1.995	7.74	28.58
45	21.53	5.30	24.6	1.605	7.45	30.28
50	26.22	6.90	26.2	2.460	9.38	35.65

<sup>1</sup> Cellulose Department, Chemical Foundation, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York.

## ISOLATION OF SUGARS FROM DEVELOPING COTTON FIBERS

After dissection, the crude cotton fibers were carefully washed in cold water to remove completely the soluble sugars. In the case of the field cotton, the volumes of these extracts were rather large due to the number of bolls collected at each stage of development. The water extracts were then diluted with twice their volume of 95 per cent ethyl alcohol and stored in tightly stoppered flasks at 0° C. until each could be analyzed. The precipitates separating at the end of this time, consisting largely of cytoplasmic protein, were removed by filtration and the clear filtrates concentrated under diminished pressure at 40° C. to thick sirups. The protein-free sirups were then weighed and portions taken for analysis.

A slightly different procedure was employed for the isolation of the sugars from the fibers of cotton bolls grown on greenhouse plants. In this case the cold water extracts of the cotton fibers were immediately concentrated under diminished pressure at 40° C. to thick sirups. The entire sirup, obtained at each stage of fiber development, was then dissolved in about 8 cc. of water and treated with calcium carbonate for the removal of substances which would interfere with the subsequent sugar determinations (1). Thus 0.1 gram of calcium carbonate was added to the sugar solution and the mixture heated in a boiling water-bath for 5 minutes. The mixture was then cooled, allowed to stand 30 minutes, again heated at 100° C. for 5 minutes, and then kept on ice for 18 to 24 hours. At the end of this time the heating was repeated as previously described. To the hot mixture there were then added 4 cc. of 96 per cent ethyl alcohol, the solution cooled and filtered into a 25 cc. volumetric flask. The filter was washed three times with 4 cc. portions of 10 per cent ethyl alcohol and the filtrate diluted to the mark. Aliquots of this solution were taken for analysis. That this procedure does not destroy or remove by adsorption any of the true sugar constituents of the fiber extracts was shown by the fact that a known mixture of glucose and fructose, invert sugar, passed through the procedure without loss in reducing power.

## NATURE OF THE SUGARS IN DEVELOPING COTTON FIBERS

The magnitude of the laevo-rotations of the sirups isolated from the cotton fibers developing in field cotton bolls (Table II) indicates mixtures of *d*-glucose and *d*-fructose. In the early stages of development, 10 to 25 days, the optical rotation of the sirups corresponds closely to that of invert sugar, -20.0°. However, in complex sugar mixtures optical rotations cannot be used alone for estimating the components.

*Identification of fructose.* The presence of fructose in the isolated sirups was qualitatively shown by the Seliwanoff test (16, 19). A small amount of the sirup was dissolved in water and a few drops of the solution added to



5 cc. of Seliwanoff's reagent (0.05 g. of resorcinol in 100 cc. of 12 per cent HCl). After heating in a boiling water-bath for about 30 seconds a red color developed and a reddish precipitate separated. Solution of the precipitate in alcohol produced a deep red color. A test, run simultaneously with invert sugar, showed no difference in behavior. All the sirups isolated from both field and greenhouse cotton gave a positive test, indicating the presence of fructose.

TABLE II  
REDUCING SUGAR CONTENT OF DEVELOPING COTTON FIBERS  
OBTAINED FROM BOLLS GROWN ON FIELD COTTON

Days after flowering	Reducing sugars in fibers			Glucose in fibers		Fructose in fibers		Pentoses in fibers		Dry fiber residue: total reducing sugars, ratio
	Wt. per boll, mg.	% wet fiber	$[\alpha]_D^{25}$ , degs.	Wt. per boll, mg.	% total sugars	Wt. per boll, mg.	% total sugars	Wt. per boll, mg.	% total sugars	
10	127.0	8.4	-15.4	45.8	36.2	64.8	51.1	16.2	12.7	100:410
15	162.4	3.7	-20.4	48.3	29.7	84.1	51.9	30.0	18.4	100:110
20	165.7	3.1	-14.2	52.0	31.4	86.3	52.1	27.4	16.5	100:110
25	111.1	2.2	-16.6	26.2	23.6	72.3	65.1	12.5	11.3	100:11.4
30	38.0	0.8	—	7.6	19.7	20.3	53.5	10.0	26.8	100:5.6
35	24.0	0.5	-37.0	4.5	18.1	10.0	42.5	9.6	39.4	100:2.7
40	5.5	0.1	-8.9	0.66	12.1	3.23	58.8	1.6	29.1	100:0.3
45	7.0	0.1	-2.7	0.83	11.9	3.85	55.0	2.3	33.1	100:0.4
50	14.4	0.5	-29.0	1.45	10.1	11.14	77.4	1.8	12.5	100:0.8

*Identification of glucose and fructose through osazone formation.* Only a few of the isolated sirups were subjected to this test, but presumably all would behave in a similar manner. As an example, 1.064 g. of the sirup isolated from 15-day cotton fibers developing in field cotton bolls (Table II) corresponding to 0.726 g. of reducing sugars, was dissolved in 50 cc. of water and 1.6 cc. (4 mol) of phenylhydrazine dissolved in 10 cc. of glacial acetic acid added. After standing for a few minutes no precipitate separated, indicating the absence of mannose, which would separate at this point (25, p. 309) as mannose phenylhydrazone had this sugar been present in the mixture. To obtain the osazone in pure condition, the solution was clarified by adding charcoal and filtering through Celite. The clear solution was then heated in a boiling water-bath for 45 minutes. After cooling the solution, the crystalline osazone separating was removed by filtration, thoroughly washed with water, triturated with methyl alcohol, filtered, and washed with methyl alcohol. Yield, 1.02 g., or 70 per cent of the theoretical. Melting point, 214°–215° C., remaining unchanged when mixed with an authentic specimen of glucose phenyllosazone.

From the optical rotations of the sirups and the yield of glucose phenyllosazone, it is apparent that both glucose and fructose are present in the solutions.

*Presence of pentoses.* The presence of pentoses in the sirups isolated from the developing cotton fiber cytoplasm was indicated by Tauber's benzidine test for pentoses (23) and Bial's orcinol test for pentoses (6). The former test is specific for pentoses whereas galactose and glycuronates, in addition to pentoses, give a positive test with the latter. However, it was possible to demonstrate the absence of glycuronates by the use of Tollens' naphthoresorcinol test (24) and the absence of galactose by failure to form galactose phenylosazone after removal of glucose and fructose by yeast fermentation. All of the sirups tested in this manner gave a positive test for pentoses.

Tauber's benzidine test for pentoses (23) was carried out as follows: one drop of a solution containing 0.05 mg. or more of pentose was boiled vigorously with 0.5 cc. of Tauber's reagent (1 g. of benzidine in 25 cc. of glacial acetic acid), and cooled. A stable cherry-red color develops. Glucose, fructose, galactose, glycuronic acids and derivatives have been found to give yellow to brown colors.

Bial's orcinol test for pentoses (6) was carried out as follows: to 5 cc. of Bial's reagent (1.5 g. orcinol and 20 to 30 drops of 10 per cent ferric chloride solution in 500 cc. of concentrated HCl) 2 to 3 cc. of the sugar solution were added and heated gently until the first bubbles rose to the surface. Immediately, or upon cooling, the solution became green. A flocculent precipitate of the same color sometimes separates.

Tollens' naphthoresorcinol test for glycuronates (24) was carried out in the following manner: to 5 cc. of the sugar solution 0.5 to 1 cc. of a 1 per cent solution of naphthoresorcinol in 95 per cent ethyl alcohol was added and then 5 cc. of concentrated HCl. The mixture was then gradually heated to boiling and allowed to boil for 1 minute while shaking the container continuously. The solution was then allowed to stand 4 minutes, cooled and extracted with an equal volume of ether. Glycuronic acids, or glycuronates, are indicated by the ether extract assuming a violet-red color. Not any of the sirups tested gave a positive reaction, although the whole fibers did give positive tests.

A summary of these results is given in Table III.

TABLE III  
IDENTIFICATION OF SUGARS OBTAINED FROM DEVELOPING COTTON FIBERS

Test	Reagent	Color	Positive + negative -	Sugar
Seliwanoff	Resorcinol + 12% HCl	Red	+	Fructose
Tauber	Benzidine + HAc	Cherry-red	+	Pentoses
Bial	Orcinol + HCl + FeCl <sub>3</sub>	Green	+	Pentoses
Tollens	Naphthoresorcinol + HCl	Violet-red	-	Glycuronic acid or derivatives
	Phenylhydrazine + HAc	Yellow ppt.	+	Glucose + fructose

QUANTITATIVE DETERMINATION OF GLUCOSE, FRUCTOSE,  
AND PENTOSE IN DEVELOPING COTTON FIBERS

The method described by Haver and Compton (12) was used for the quantitative determination of glucose, fructose, and pentoses in the sirups isolated from the cotton fiber cytoplasm.

Briefly, the isolated sirups or prepared solutions previously described were dissolved or diluted to a volume such that the resulting solution contained about 2 mg. per cc. of reducing sugar. The analysis was then resolved into three steps:

(A) The total reducing sugars in 1 cc. of the solution were determined using the Hanes modification (11) of the Hagedorn-Jensen procedure, in conjunction with the formula of Hulme and Narain (14),  $\text{sugar (mg. per cc.)} = b (T_2 + a)$ . In this formula,  $T_2$  is the titration difference in cubic centimeters of 0.01 N sodium thiosulphate solution,  $a$  is a constant whose value is 0.05 cc., and  $b$  is a factor whose value depends upon the reducing power of the various sugars. With the original sugar solution the value of  $b$  was found to be equal to that of glucose and fructose, namely, 0.338, but after removal of these constituents by yeast fermentation, (C) below, the pentose value 0.368 was employed.

(B) Aldose sugars (glucose + pentoses) were determined in either of two ways. (a) Thirty cc. of the sugar solution were tested by the Willstätter and Schudel hypiodide procedure (26). In the equation, aldose sugar (mg. per cc.) =  $C \times T / 30$  cc.,  $T$  is the corrected titration in cc. of 0.1 N iodine solution, and  $C$ , a factor determined for each sugar, i.e., for glucose, 9.00 mg., per cc., and for pentoses, 7.505 mg. per cc. Unless the proportion of these two aldose sugars is known, a quantitative determination at this point is impossible. This titration will accordingly be represented by  $B$ . (b) Using the MacLeod and Robison (17) adaptation of the iodimetric method to microtechnique, 2 cc. of the solution were tested. Since 0.005 N iodine solution was used, it was necessary to convert one-half the titration obtained by this procedure into the equivalent of 0.1 N iodine solution to obtain milligrams of aldose sugars per cubic centimeter.

(C) The hexoses (glucose + fructose) were removed from a 1 cc. aliquot of the sugar solution, after diluting to 10 cc. with distilled water, by adding 3 cc. of a washed yeast suspension (12). After standing with occasional shaking for three hours the solution was filtered through Celite and analyzed as described in step (A) above for remaining reducing sugars. The titration value after correction was divided by 0.85, since 15 per cent of the pentoses were removed during the three-hour period of fermentation.

The quantitative relation between the three sugar components is calculated from the titration values obtained above, taking into consideration the total volume of the solution from which aliquots were taken. Thus,  $C$ , pentose (mg. per cc.) =  $0.368 (T_1 + a)$  where  $T_1$  is the corrected titration





[see (A) above] for remaining reducing sugars after removal of glucose and fructose.  $A$ , total reducing sugars (mg. per cc.) =  $0.338 (T_2 + a)$  where  $T_2$  is the corrected titration for total reducing sugars. Now the difference between these two values  $A - C$  = (mg. per cc.) of glucose + fructose in the solution. The pentose value, in mg. per cc., may now be converted into the equivalent titration of pentoses expressed in cubic centimeters of 0.1 N iodine solution, using the equation, pentoses (mg. per cc.) =  $7.505 \times T_3$ , where  $T_3$  is the unknown pentose titer which is thus determined. Since  $B$  is the titration value of glucose ( $T_4$ ) + pentoses ( $T_3$ ), in cubic centimeters of 0.1 N iodine solution, subtraction of the corresponding calculated pentose titer from  $B$  gives that part of the aldose titration due to glucose ( $T_4$ ). This titration value is then substituted in the equation, glucose (mg. per cc.) =  $9.00 \times T_4$ . The value of glucose thus determined when subtracted from  $A - C$ , or glucose + fructose, in mg. per cc., gives the fructose content in mg. per cc.

The results obtained upon applying this method of analysis to the sirups isolated from the developing cotton fiber are given in Tables II and IV.

#### RESULTS AND DISCUSSION

In the first publication of this series (7) the relation between certain basic components of the developing cotton fiber was shown. The components arbitrarily chosen were dry fiber residue, total reducing sugars in the fiber, fats and waxes soluble in alcohol-benzene, and total fiber moisture. The cotton bolls selected for these determinations were obtained from different cotton plants. For the purpose of correlating the data subsequently obtained on the composition of the cotton fiber reducing sugars, the average weights and percentages of the fibrous constituent of cotton bolls obtained from a single cotton plant grown in the greenhouse are given in Table I. Upon comparing these data with those previously obtained (7) from different cotton plants, little difference in trends can be noted.

Qualitative tests on the sirups isolated from the cotton fibers at various stages of development show the presence of glucose, fructose, and pentoses (Table III). The negative test for glycuronic acids in the sirups would seem to indicate that the polyuronides are located principally in the cell wall and do not exist to any appreciable extent as entities of low molecular weight in the fiber cytoplasm. It is possible, however, that if such compounds had been present in the fiber cytoplasm they would have been removed from the sirups during the isolation processes employed. As will be shown in a later publication, very little of the total high molecular weight polyuronides present in the cotton fiber are removed by washing with cold water or exist free in the fiber cytoplasm.

Applying the method of Haver and Compton (12) to the analysis of the sirups isolated from the cotton fibers, the quantitative relation between the

glucose, fructose, and pentose constituents at various stages of development was determined (Tables II and IV). As previously shown (7) during the early stages of development, cotton fibers obtained from bolls grown on greenhouse cotton plants contain a larger amount of reducing sugars per boll than the fibers obtained from bolls grown on field cotton plants, although the percentages of reducing sugars present on the basis of the fresh wet fiber weight are approximately the same. A similar relation also exists with each of the components of the isolated sirups as shown by comparing the analyses of the cotton fiber sirups obtained from bolls grown on greenhouse and field cotton plants. The advantage of obtaining cotton bolls from the same plant for these determinations is clearly shown (Table IV). The greater uniformity of these analyses would seem to show that the relative amounts of the various sugars in the cotton fibers vary from plant to plant at comparable stages of development but are fairly uniform for a particular plant. Due to the composite nature of these results, therefore, it is not safe to draw conclusions regarding the carbohydrate metabolism of a single fiber.

Cellulose in the form of microscopic particles ( $1.1 \times 1.5\mu$ ) have been observed in the cotton fiber as soon as cell elongation begins (8, 9, 20). As fiber elongation proceeds these elementary units appear to undergo end-to-end alignment to form the fibril structure of the cotton fiber (9). Cotton fiber elongation continues until about the twenty-fifth to thirtieth day with little or no change in cell diameter (2, 3, 13). During the next 20 to 25 days the cell wall thickens by the deposition of successive layers or lamellae of cellulose with other cell constituents (2, 4) and the characteristic spiral arrangement of the fibrils becomes evident (5, 20). At the beginning of this latter period, particularly as shown in the case of field cotton (Table II) and somewhat in the case of greenhouse cotton (Table IV), an abrupt lowering of the amount of reducing sugars per boll occurs. That there is no preferential utilization of any one of the sugars present in the mixture is definitely shown. It appears therefore that glucose, fructose, and the pentoses are converted into the various cell wall constituents at approximately the same rate during the active period of fiber development or that these sugars are all mutually interconvertible.

The relation between the crude dry fiber and total reducing sugars is better shown in the form of the ratio, dry fiber residue: total reducing sugars (last column, Tables II and IV). It will be noted that the greatest lowering of the total reducing sugar component of the ratio occurs between the tenth and fifteenth day, i.e., during the period of rapid fiber elongation. A second lowering, which is presumably a continuation of the first, of smaller magnitude, occurs about the twentieth day. Thereafter the amount of sugar present relative to the fiber mass decreases steadily. These data are in

agreement with what would be expected from the present knowledge concerning the development of the cotton fiber (5, 9, 15, 20).

The pentose component of the reducing sugars of the developing cotton fiber is of considerable interest. Spoehr (21, 22) has called attention to the importance of the pentoses and pentosans in the activity of plant cells, and from the present analyses the pentoses appear to be a necessary constituent of the developing cotton fiber. In general, the reducing sugars of cotton fibers obtained from bolls grown on field plants contain a higher percentage of pentoses than the reducing sugars of cotton fibers obtained from bolls grown on greenhouse plants. Beginning with the fifteenth day after flowering, in the case of bolls grown on the same plant, the pentose content of the cotton fibers varies within the very narrow limit of 5 to 10 per cent of the total reducing sugars (Table IV). On the other hand, when the bolls are obtained from different plants, the pentose content of the fibers fluctuates between rather wide limits without developing any definite trends (Tables II and IV). Neither the various pentoses in the mixture nor the relative amounts of each have as yet been determined. From previous work on plant materials, the more probable pentoses (18, p. 184) are (a) *d*-xylose, which is usually associated with cellulose, (b) *l*-arabinose, associated with pectins, and (c) *d*-ribose, which is present in plant nucleic acids. The pentose component is being further investigated.

#### SUMMARY

The reducing sugars of developing cotton fibers are composed of glucose, fructose, and pentoses. Throughout the period of fiber development the sugars are present in variable relative proportions. During the period of rapid cell wall elongation, 1 to 20 days, the reducing sugar component is high relative to the mass of the crude fiber, but progressively decreases during cell wall thickening, "secondary wall formation," until maturity is reached. The mechanism of the transformation of the various sugars into the many cotton fiber constituents lies as yet in the realm of speculation.

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## FACTORS FOR COLOR IN THE PRODUCTION OF POTATO CHIPS

F. E. DENNY AND NORWOOD C. THORNTON

This problem was brought to our attention by the research department of a food distributing company whose representatives reported difficulty in obtaining potato tubers that would furnish chips of a suitable color, especially in late winter and early spring.

Although the relation of storage temperature to the sugar content of the tubers had already been established (5, 8, 3), and although high sugar content had been shown by Sweetman (6) to be the cause of the dark-brown color so objectionable in potato chips, some preliminary experiments were undertaken, especially from three points of view: (a) the behavior of different varieties, since this factor had not been studied extensively in previous experiments; (b) the effect of a previous storage temperature upon the sweetening system in the tuber, since Barker (2) had suggested that even a short storage period at 15° C. retarded subsequent sweetening; (c) the possibility of using the vapor of the methyl ester of naphthaleneacetic acid to inhibit sprouting (Guthrie, 4) and therefore to permit storage at temperatures favorable for maintaining a low sugar content in the tubers.

The results of tests on sugar content and potato chip color of tubers of eleven varieties of potatoes (*Solanum tuberosum* L.) of the 1939 crop when tubers were subjected to different storage conditions during the period from December 1939 to May 1940 are dealt with in this paper, and the effect of the vapor of methyl ester of naphthaleneacetic acid upon the sprout development and sugar content of tubers of these varieties is to be reported upon in a paper to appear subsequently in the Contributions from Boyce Thompson Institute.

There was early recognition of the fact not previously noticed that, at the temperature used in making potato chips, only the reducing sugar content, not that of the sucrose, was responsible for the brown color of the chips (Thornton, 7). As a result of this observation the experimental procedure for this and especially for future tests together with the problem of reporting results were greatly simplified.

There were large differences among the varieties with regard to the reducing sugar content and the color of chips. Indeed, one variety, Russet Rural, gave chips that under most of the storage conditions were too light-colored, or even ivory-colored; and from another variety, Bliss Triumph, chips satisfactory in color could not be obtained. However, these results rep-

resent merely those obtained in a single year from tubers largely from a single locality, and to what extent they represent variety characteristics and to what extent responses to a particular environment can be determined only by additional tests.

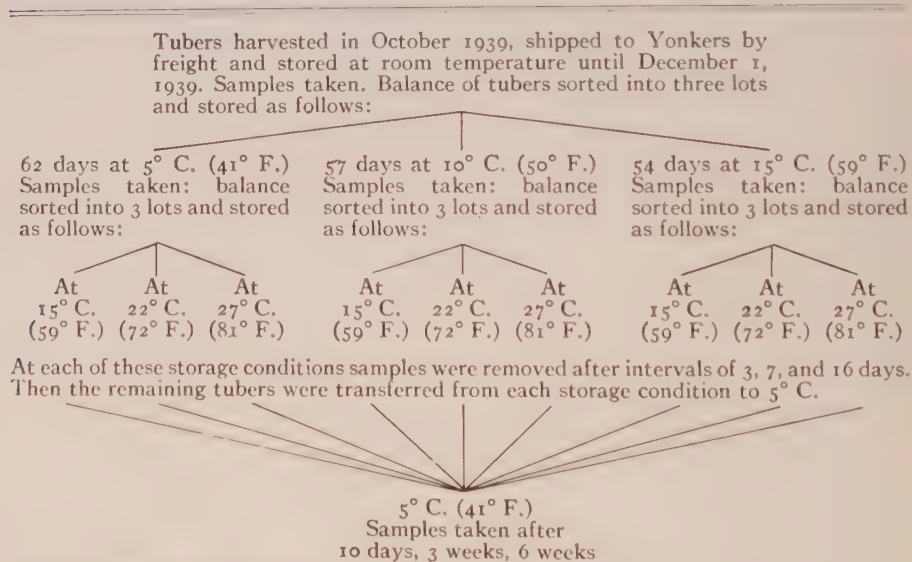
Confirmation was obtained of previous reports that storage at a temperature as low as 5° C. (41° F.), at which sprouting was inhibited, induced such a high sugar content in the tubers that the color of the chips was unsatisfactory; and also that at a temperature of 10° C. (50° F.), if continuously maintained, sprouting ensued freely in all of the varieties tested. Nevertheless, the gradient between 5° and 10° was sharp, and it seemed not impossible, especially in view of the large differences noted in the behavior of different varieties, that at some intermediate temperature tubers of one or more varieties could be held for long periods without either undue sprouting or accumulation of reducing sugar.

#### METHODS

*Storage and sampling.* The scheme for the storage of the tubers and for the removal of samples for analysis or production of potato chips is shown in Table I. Samples were taken first at the time the tubers were put into

TABLE I

SCHEME SHOWING CONDITIONS OF STORAGE AND TIME OF SAMPLING FOR DETERMINATION OF REDUCING SUGAR AND FOR PRODUCTION OF POTATO CHIPS



Note: All tubers harvested from fields in north-central New York State except those of the variety Chippewa, which were supplied through the courtesy of Eastern States Farmers Exchange, Springfield, Mass.

storage. The tubers to be stored were sorted evenly into paper bags representing as many lots as were scheduled for removal during the course of the tests, the bags being numbered and dated to facilitate the sampling. The lots at 5° C., 10° C., and 15° C. were in a thermostatically controlled room in which the fluctuations of temperature were very small, but the lots listed in the tables as at 22° C. (72° F.) were in reality at room temperature which during the course of the tests was at approximately this value but fluctuated from this temperature by approximately  $\pm 2^{\circ}$  C. Data in the tables in this paper show that such small differences in temperature in this particular range do not have any important influence on the reducing sugar content of potato tubers or, consequently, on the color of potato chips.

It will be noted in Table I that after a period of storage at the temperatures of 5°, 10°, and 15° C. tubers were transferred to higher temperatures in order to induce a decrease in sugar, and samples were taken at successive intervals thereafter. After the storage interval at these higher temperatures had reached 16 days the tubers that had not been used in running samples were again distributed by lot into samples which were placed in cold storage at 5° C. to remain until samples were to be removed at intervals of 10 days, 3 weeks, and 6 weeks.

Twelve tubers were taken for a sample and these furnished juice for the sugar determinations and slices for the potato chips.

*Preparation of potato chips.* Transverse sections, approximately one-half inch thick, were cut from the middle portion of each tuber, peeled as thinly as possible, and dropped into cold water. The sections were next passed over the cutting knife of a vegetable slicer so adjusted that slices about one-sixteenth of an inch in thickness were cut from each piece of potato according to the method of Sweetman (6). The first few slices were discarded and then usually two slices to be cooked were cut from each section. After slicing, the chips were washed with cold water to rid them of free starch granules and they were soaked for about fifteen minutes with occasional rinsing in cold water. The slices were next laid between paper towels to rid them of excess moisture before being cooked. The cooking medium was pure lard, 1.5 lbs. in a vessel 10 inches in diameter at top (tapering to 6.5 inches at base) and 4 inches deep, heated to a temperature of 195° C. (383° F.) at the time the chips were introduced which cooled to a temperature of 158° C. to 166° C. (316° F. to 331° F.) just previous to the time at which the chips were removed. The cooking period of different lots varied because of differences in sizes of the chips but in every case it was found that the chips were properly cooked when the water was removed. It was at the stage of cooking when bubbling ceased, indicating the removal of water, that the chips were considered sufficiently cooked. During the cooking period the chips were stirred constantly to insure proper cooking on both sides. Duplicate and triplicate lots of chips from the same potatoes

were readily cooked to the same depth of color, indicating the uniformity of the procedure.

*Sugar analyses.* The remaining portions of the tubers, after removal of the transverse sections for chipping, were quartered longitudinally and a quarter from each tuber was passed through a food chopper. The juice was squeezed from the finely ground tissue and a 25 cc. sample was taken, neutral lead acetate was added, the volume made up to 200 cc., filtered, and delead with sodium oxalate. Reducing sugars were determined on a 50 cc. aliquot of this prepared sample according to the Munson and Walker method (1, p. 379-381), and the cuprous oxide was titrated with a potassium permanganate solution which had been standardized with a sugar solution of known concentration. Another 50 cc. aliquot of the sample was hydrolyzed overnight with 5 cc. HCl (1, p. 373), then neutralized and made up to 100 cc. Reducing sugars were determined on a 50 cc. aliquot of this hydrolyzed sample and the sucrose was determined by difference. Both the reducing sugars and sucrose content of these potatoes were expressed as mg. per cc. of juice.

It is realized that in nearly all of the previous reports with regard to sugar content of tubers used for potato chips, the data are given on the basis of the weight of tissue rather than of the volume of juice. Because of the large number of analyses to be made in this experiment, and the need of having an immediate measurement of the sugar for comparison with the chips made from the same tissue, it was necessary to deal with juice rather than tissue. However, in order that values here reported on the basis of mg. per cc. of juice may be compared with previous values on the basis of mg. per g. of tissue, a test was made on four different lots using the same portion of minced tissue for determinations by both methods. The values expressed on the mg. of reducing sugar per cc. of juice for samples from the varieties Carman No. 3, Spaulding Rose, Irish Cobbler, and Green Mountain were: 6.7, 16.3, 2.1, 5.0; and the corresponding values for these same lots in the same order on the basis mg. per g. of fresh weight of tissue were: 5.1, 13.6, 1.9, 3.8; the ratios of tissue to juice values are therefore: 0.76, 0.84, 0.90, 0.76, with an average value of 0.82. Therefore, values given as mg. per cc. of juice may be converted into values as mg. per g. of tissue, at least for purposes of approximate comparison by multiplying by 0.8.

## RESULTS

### TEMPERATURES DURING COOKING

Although the starting temperature of the fat for cooking potato chips was uniformly 195° C. (383° F.) for the routine tests throughout this experiment, temperatures both higher and lower were tried and the results are shown in Table II. The sample consisted of 20 chips in each case and the weight was approximately 42 grams. Table II shows the reading at suc-



TABLE II  
TEMPERATURES OF THE COOKING FAT AT INTERVALS AFTER THE START

Seconds after start of cooking	Temperatures of the cooking fat when the starting temperature was				
	150° C. (302° F.)	175° C. (347° F.)	195° C. (383° F.)	220° C. (428° F.)	240° C. (464° F.)
0	150	175	195	220	240
10	145	165	181	208	—
20	142	157	169	190	222
30	138	151	164	180	203
40	136	149	160	—	190
50	134	146	158	175	194
60	133	145	158	175	—
75	133	145	161	180	198
90	133	147	166	185	—
105	—	154	169	—	—
120	135	158	—	—	—
135	140	—	—	—	—
150	145	—	—	—	—

cessive intervals of a thermometer with bulb immersed in the constantly-stirred fat.

Toward the end of each period of cooking it will be noted that the temperature rose; this occurred at the time that the water was practically completely removed from the tissue as shown by the gradual cessation of bubbling. The chips were removed at the time indicated by the last entry in each column, which coincided with the complete cessation of bubbling.

It was observed that the development of color occurred at about the time or just preceding this rise in temperature. After this brown color once developed it was stable for many seconds, and in order to obtain any important increase in browning it was necessary to allow the chips to remain in the fat for at least one minute after this end point of color was reached.

These critical temperatures at which color was developed were important for establishing the temperatures to be used in the preparation of artificial "potato chips" using filter paper impregnated with sugars and starch.

#### FILTER PAPER "CHIPS"

In the early stages of the tests, in comparing the color of potato chips with the reducing sugar, sucrose, and total sugar values of the juice obtained from the tubers which furnished the chip samples, it was noted that the color correlated well with the reducing sugar, less satisfactorily with the total sugar, and not at all with the sucrose values.

Models of chips were made by soaking pieces of filter paper in dextrose and sucrose solutions and in a potato starch suspensiom, stapling several of these papers together, and drying the small discs. These samples were dropped into hot fat and were cooked in a manner corresponding to that for potato chips. Since these models had very little water in them, a defi-

nite temperature was selected for the cooking and the duration of the cooking was regulated for the same period of time at each of the temperatures used for cooking the corresponding lots of potato chips in Table II. The temperatures selected were 140°, 154°, 166°, 180°, and 190° C., corresponding to what appeared to be the critical temperatures for color development as shown in the columns in Table II.

The results obtained with these models in the case of four of these temperatures are shown in the right-hand column in Figure 1. At a cooking temperature of 166° C., corresponding to a starting temperature of 195° C., such as was used in preparing chips from potato tubers, color was obtained with papers prepared from dextrose but not from those prepared from sucrose or starch. When, however, the cooking temperature was higher, i.e., about 180° C., corresponding to a starting temperature of 220° C., some browning occurred with sucrose, and at the temperature of 190° (starting temperature 240° C.) the color with sucrose became dark brown.

Further evidence that the sucrose content of the tubers is not a factor for browning in potato chips prepared under these conditions with a starting temperature of 195° C. (383° F.) is shown in Figure 1 Column 4. No correlation between the sucrose in the juice and the color of the chips made from the tubers was found.

This does not mean that the sucrose content cannot be a factor in browning. It can if the temperature of the cooking fat is sufficiently high. For example, in the present tests tubers of Russet Rural usually furnished chips too light in color, but these same tubers gave chips of a satisfactory brown color if the starting temperature of the fat was 220° C. (428° F.). The sucrose content of Russet Rural tubers was relatively high in these tests and no doubt at this higher temperature the sucrose was attacked and browning resulted.

*Tests with other sugars.* Filter paper tests were made in the same manner with other sugars. The monosaccharides, arabinose and levulose, developed color to the same extent as dextrose when heated at the different temperatures. The disaccharides, lactose and maltose, were next tested with the result that lactose developed only about one-half the depth of color as shown by sucrose in Figure 1 at any of the cooking temperatures. Maltose on the other hand developed a brown color much more rapidly at the start of the heating in the fat, but in every case these filter papers developed the same depth of color as dextrose.

#### POTATO CHIP COLOR AND SUGAR CONTENT

The experiment on the storage of tubers of eleven different varieties at different temperatures for different periods of time involved 57 samples of each variety and thus furnished 627 samples of potato chips with the corresponding values for reducing sugar and sucrose from the analyses of the juices.



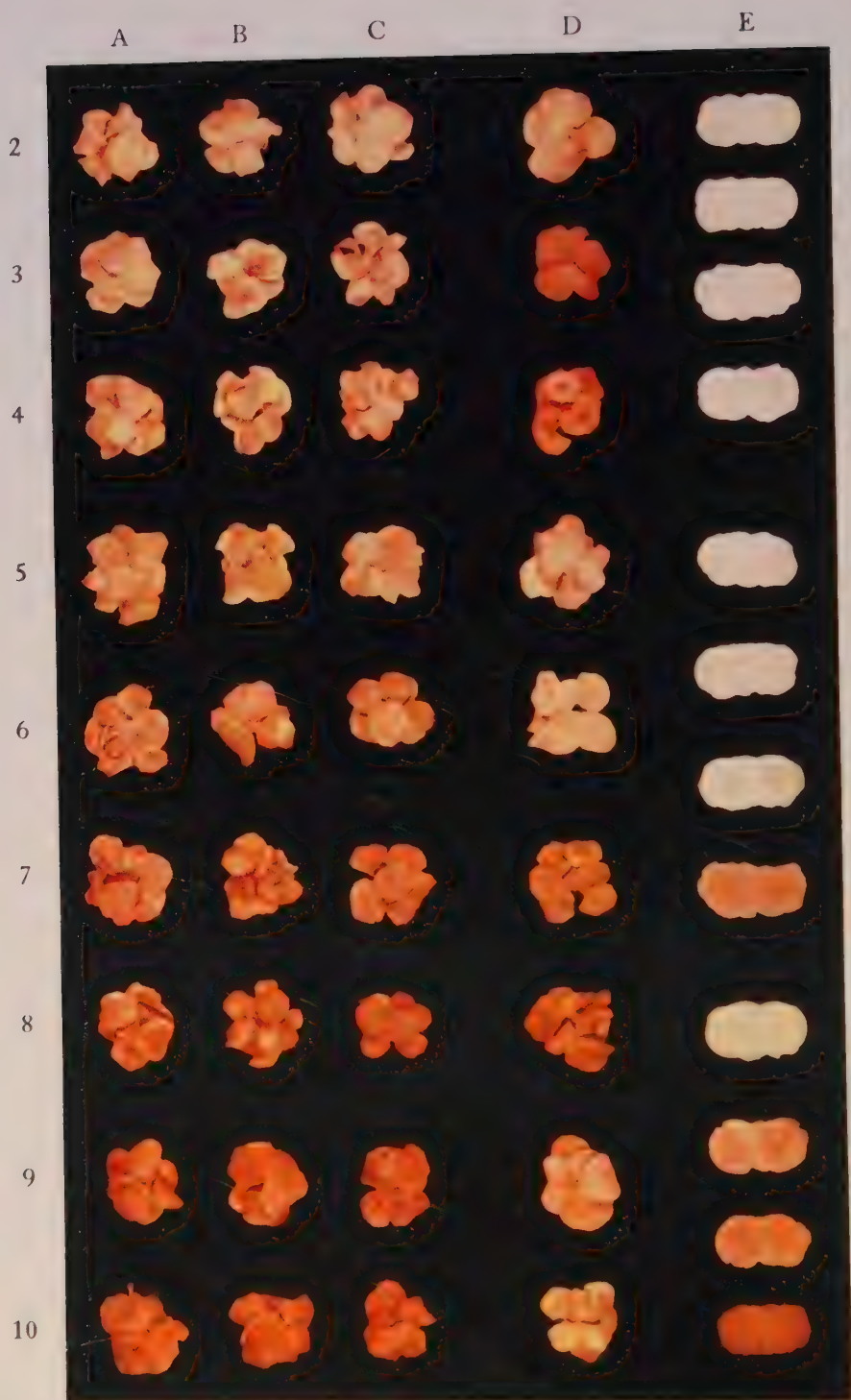


FIGURE 1.



# EXPLANATION: FIGURE 1

The values at the left edge, i.e., 2, 3, 4, etc. are milligrams of sugar per cc. of juice from potato tubers from which the potato chips shown in Columns A, B, C, and D were prepared. For Columns A, B, and C these values 2, 3, 4, etc. are for reducing sugar; for Column D they are for sucrose. In Column E are photographs of "chips" made by soaking filter papers in sugar solutions and a starch suspension and then immersing the papers in hot fat in a manner comparable to that used in making potato chips. Top four lots in Column E, filter papers soaked in potato starch suspension; middle four, filter papers soaked in sucrose solution; bottom four, filter papers soaked in dextrose solution. Temperatures of cooking fat for the four lots reading from top to bottom in each of the three sets were: 154° C., 166° C., 180° C., 190° C. The temperature at 166° C. corresponds to a starting temperature of 195° C. (383° F.) in cooking potato chips, and was the temperature used in the routine potato chip preparations made in these experiments.

SUGAR VALUES OF THE SAMPLES OF POTATO CHIPS IN FIGURE 1, COLUMNS A, B, C, AND D.  
MILLIGRAMS PER CC. OF JUICE

Reducing Sugar				Sucrose				Total Sugar			
A	B	C	D	A	B	C	D	A	B	C	D
2.0	2.0	2.0	3.5	3.5	5.2	1.9	2.0	5.5	7.2	3.9	5.5
3.0	3.0	2.9	10.3	3.2	10.6	2.3	3.0	6.2	13.6	5.2	13.3
4.0	4.0	3.9	7.6	3.3	4.6	2.9	3.9	7.3	8.6	6.6	11.5
5.1	5.1	5.0	2.9	1.4	2.9	2.1	5.0	6.5	8.0	7.1	7.9
6.0	6.0	6.0	0.0	4.2	3.5	4.2	6.1	10.2	9.5	10.2	6.1
7.0	6.9	7.1	8.6	1.8	7.4	6.9	7.0	8.8	14.3	14.0	15.6
8.0	8.2	7.9	9.8	3.0	2.7	2.1	8.2	11.0	10.9	10.0	18.0
9.0	8.9	9.2	1.8	2.1	4.0	7.0	9.1	11.1	12.9	16.2	10.9
10.0	10.0	10.1	0.2	3.9	4.3	2.4	9.9	13.9	14.3	12.5	10.1

VARIETIES REPRESENTED IN POTATO CHIP LOTS IN FIGURE 1

A	B	C	D
Russet Burbank	Carman No. 3	Chippewa	Irish Cobbler
Blue Victor	Russet Rural	Irish Cobbler	Spaulding Rose
Green Mountain	Carman No. 3	Russet Burbank	Katahdin
Spaulding Rose	Blue Victor	Russet Burbank	Chippewa
Green Mountain	Spaulding Rose	Early Ohio	Russet Rural
Blue Victor	Early Ohio	Spaulding Rose	Bliss Triumph
Green Mountain	Early Ohio	Russet Burbank	Irish Cobbler
Spaulding Rose	Green Mountain	Spaulding Rose	Katahdin
Green Mountain	Bliss Triumph	Early Ohio	Carman No. 3

STORAGE CONDITIONS REPRESENTED IN FIGURE 1

Storage condition				Place of sample in Figure 1	
62 days at 5° C.	+ 3 days at 15° C.			3D	
" " " " " " " " " "	" " " " " " " " " "			4D, 10A, 8C, 10C	
" " " " " " " " " "	" " " " " " " " " "			2C, 7A	
" " " " " " " " " "	" " " " " " " " " "			9A, 8B	
" " " " " " " " " "	" " " " " " " " " "			4A, 10B	
" " " " " " " " " "	" " " " " " " " " "			6B	
" " " " " " " " " "	" " " " " " " " " "			6A	
" " " " " " " " " "	" " " " " " " " " "			7D	
" " " " " " " " " "	" " " " " " " " " "			0B, 6C	
" " " " " " " " " "	" " " " " " " " " "	+ 10 days at 5° C.		5D	
" " " " " " " " " "	" " " " " " " " " "	+ 3 weeks " " " "		4B	
" " " " " " " " " "	" " " " " " " " " "	+ 6 " " " "		3C	
57 " " " " " " " " " "	" " " " " " " " " "			8A, 2D	
" " " " " " " " " "	" " " " " " " " " "			5A	
" " " " " " " " " "	" " " " " " " " " "			5C	
" " " " " " " " " "	" " " " " " " " " "			4C	
" " " " " " " " " "	" " " " " " " " " "			2A	
" " " " " " " " " "	" " " " " " " " " "			2B, 5B	
" " " " " " " " " "	" " " " " " " " " "	+ 3 weeks " " " "		3A	
" " " " " " " " " "	" " " " " " " " " "	+ 10 days " " " "		7C	
" " " " " " " " " "	" " " " " " " " " "	+ 3 weeks " " " "		8D	
" " " " " " " " " "	" " " " " " " " " "	+ 6 " " " "		6D	
54 " " " " " " " " " "	" " " " " " " " " "	+ 10 days " " " "		9C	
" " " " " " " " " "	" " " " " " " " " "	+ 6 weeks " " " "		10D	
" " " " " " " " " "	" " " " " " " " " "	+ 10 days " " " "		7B, 9D	
" " " " " " " " " "	" " " " " " " " " "	+ 3 weeks " " " "		3B	
" " " " " " " " " "	" " " " " " " " " "	+ 6 " " " "			

The relation between the sugar content of juices and the color of the chips made from the tubers which furnished the juices is shown in Figure 1. The samples used for the original "Kodachrome"-photograph were selected by lot in the following way: From a chart showing all of the sugar analyses a sample number was called off when one was found that had a reducing sugar content of 2.0 mg. per cc. of juice; this is the sample at the top of Column A opposite the value 2. Again a selection was made of a sample showing a reducing sugar value of 3.0 mg.; this is the second lot from the top in Column A. In this manner the other lots in Column A and those in Columns B and C were obtained. The lots in Column D were selected in the same way except that the values 2, 3, 4, etc. refer to sucrose rather than to reducing sugar.

The only selectiveness that deviated from complete randomness in the choosing of these representative samples was that the choice was spread deliberately to include samples from all of the varieties, and a value from one variety having been taken, the next selection was sought among the values from some other variety. In some cases a value exactly 2, 3, 4, etc. could not be found and a value as close as possible to it was taken, differing in most cases from the whole number by only 0.1 or 0.2.

The chip samples shown in Figure 1 Columns A, B, and C represent a series of reducing sugar values ranging from 2 to 10 mg. of reducing sugar per cc. of juice. It is seen that there is a gradual darkening of color in the order top to bottom. The dividing line beyond which the color would be regarded as too dark for satisfactory chips is a matter for individual preference. In our opinion it is at about 5 mg. of reducing sugar per cc. of juice. This would be equivalent to about 4 mg. of reducing sugar per g. of fresh tissue.

The samples in Figure 1 Column D are selections made in the same way on the basis of the *sucrose* content of the juice. The values 2, 3, 4, etc. here also refer to the number of mg. per cc. of juice, except that in this column the values refer to sucrose instead of reducing sugar. It is seen that a gradation with respect to sucrose values does not give a gradation with respect to color of chips.

The legend for Figure 1 gives data for the reducing sugar, sucrose, and total sugar values for each of the chip samples in Columns A, B, C, and D. The total sugar values show a fair correlation with the color development but in many cases the total sugar value is out of the order expected from the color shown. This is due to variations in the sucrose values which influence the total sugar value of the juice but not the color of chips, at least under the conditions for cooking used in these tests.

The legend for Figure 1 also emphasizes the large number of storage conditions that were involved in the preparation of the samples shown in Columns A, B, C, and D. Thus, the potato chip samples shown in Figure 1

represent eleven varieties and 26 different storage conditions. This emphasizes to what extent the reducing sugar content is determinative of the color of the chips. Whatever the variety and the storage condition, the amount of reducing sugar is the critical factor for the color of the chips.

REDUCING SUGAR CONTENT OF JUICE OF TUBERS OF DIFFERENT  
VARIETIES STORED AT VARIOUS TEMPERATURES

The effect of storage temperature upon the reducing sugar content of tubers of the eleven different varieties is shown in Tables III and IV.

*Continuous storage at room temperatures.* Column 2 in Table III shows the reducing sugar on January 9 of the samples that had been allowed to remain at room temperature (approximately 22° C. or 72° F.). Although these tubers had not been in cold storage and had been exposed to only such low temperatures as had occurred in nature from the time of harvest until their arrival in Yonkers in mid-November the values in Column 2 show high sugar values for Bliss Triumph and Green Mountain, and these lots of tubers of these two varieties were found unsuitable for potato chips even under these favorable temperature conditions of storage.

*Continuous storage at low temperatures.* Columns 3, 4, 5, Table III, show the reducing sugar values after 62 to 54 days of storage at the temperatures of 5°, 10°, 15° C. The unsuitability of a storage temperature as low as 5° C. (41° F.) even for such a short period is apparent, since all varieties except one show reducing sugar values higher than 5.0 mg. per cc. of juice (which is regarded in this report as the dividing line of sugar content for good color in chips). When stored at 10° C. (50° F.), tubers from five of the varieties, Bliss Triumph, Blue Victor, Green Mountain, Russet Burbank, and Early Ohio, were too high in reducing sugar for chips of satisfactory color. It is true that upon long storage of tubers at 10° C., i.e., from December until May, the reducing sugar values (not shown in this report) were lowered to the place at which good chips were obtainable from all varieties except Bliss Triumph, but such extensive sprouting had occurred by that time that storage at such a temperature was not regarded as feasible. The sugar values after storage at 15° C. (59° F.) for 54 days are shown in Column 5, Table III, and although the reducing sugar values are not much lower at 15° than at 10°, sprouting was much more extensive at 15° than at 10°.

These results agree with those of previous experimenters (5, 8) in indicating that continuous storage at a temperature which is low enough to inhibit sprouting results in the development of sufficient sugar to cause too dark a color in the chips. However, some possibility is indicated that with such varieties as Russet Rural, Carman No. 3, Irish Cobbler, Chippewa, and Katahdin a temperature somewhere between 5° and 10° might be satisfactory for retarding sprouting and at the same time be too high for ex-

TABLE III  
REDUCING SUGAR CONTENT OF JUICE OF POTATO SAMPLES USED FOR PRODUCING POTATO CHIPS

Variety	Milligrams of reducing sugar per cc. of juice												
	After storage from Nov. 24 to Jan. 9 at room temp.	After storage which started on Dec. 1 and continued for			After removal from 5°, 10°, and 15°, then after storage for 16 days						At 27° C.		
		62 days at	57 days at	54 days at	At 15° C. after removal from			At 22° C. after removal from			after removal from		
					5° C.	10° C.	15° C.	5° C.	10° C.	15° C.	5° C.	10° C.	15° C.
Russet Rural	0.0	4.1	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chippewa	0.0	6.6	2.4	2.1	1.7	1.2	1.8	1.3	0.0	0.0	0.0	1.6	1.1
Katahdin	0.0	9.8	4.0	1.0	2.9	2.6	2.3	1.6	0.0	0.0	0.0	1.3	1.1
Carman No. 3	1.1	7.3	1.5	1.4	2.9	1.1	1.1	0.0	0.2	2.3	0.0	0.0	0.0
Irish Cobbler	2.2	8.3	2.2	2.3	4.7	0.7	2.6	0.0	1.3	2.3	0.0	0.0	0.0
Early Ohio	2.7	9.6	5.1	3.2	2.6	3.0	5.1	2.5	0.9	1.6	2.3	2.0	1.8
Russet Burbank	2.7	11.1	5.8	4.2	5.9	2.8	4.6	3.9	1.0	2.3	2.4	2.0	1.8
Blue Victor	3.0	13.0	6.9	3.4	4.3	2.3	5.3	2.3	2.0	4.2	2.0	2.6	2.3
Spaulding Rose	3.6	13.7	6.3	4.6	8.0	3.4	5.8	4.4	3.6	4.1	2.6	3.4	2.3
Green Mountain	5.9	13.5	6.6	6.1	5.9	3.6	4.0	4.6	4.2	5.8	3.4	2.4	2.4
Bliss Triumph	7.3	20.4	7.9	6.1	10.8	6.4	10.0	7.6	5.9	8.6	6.9	4.3	4.3

Note: The entry 0.0 means the amount was less than 0.2 mg. per cc. of juice.



cessive sugar formation. This point needs further testing with tubers of these and other varieties from various sources.

*Transfer from low to higher temperatures.* The effect upon the reducing sugar content of tubers brought about by the transfer of the tubers from low to higher temperatures for a short period (in this case, for 16 days) is shown in Table III, Columns 6 to 14. This transfer to higher temperatures caused a reduction in the amount of reducing sugar even when the higher temperature was as low as 15° C., and particularly when it was 22° C. (room temperature) or 27° C. The most important consideration here is what value of reducing sugar had been attained after the transfer from 5°

TABLE IV

REDUCING SUGAR VALUES OF JUICES OF TUBERS RETURNED TO COLD STORAGE  
AFTER A TEMPORARY PERIOD AT HIGHER TEMPERATURES

Variety	Milligrams of reducing sugar per cc. of juice								
	Tubers transferred to 5° C. for 3 weeks after having been at 15° C., 22° C., and 27° C. for 16 days								
	Lots at 5° C. for 62 days before an inter- mediate 16-day period			Lots at 10° C. for 57 days before an inter- mediate 16-day period			Lots at 15° C. for 54 days before an inter- mediate 16-day period		
	At 15° C.	At 22° C.	At 27° C.	At 15° C.	At 22° C.	At 27° C.	At 15° C.	At 22° C.	At 27° C.
Russet Rural	1.6	0.3	0.2	0.6	0.0	1.9	0.0	0.2	2.4
Chippewa	2.9	3.1	2.9	3.0	2.4	3.1	3.1	2.3	3.0
Katahdin	7.4	4.9	3.6	4.7	3.6	2.9	1.4	1.8	2.3
Carman No. 3	4.6	2.1	2.9	2.0	1.6	2.6	3.0	3.6	4.1
Irish Cobbler	6.7	3.2	4.3	2.3	1.6	2.7	3.1	3.8	4.1
Early Ohio	9.0	5.8	8.3	5.6	5.1	7.4	3.9	6.9	8.4
Russet Burbank	10.2	7.8	7.7	6.4	5.8	5.8	4.3	4.4	5.5
Blue Victor	5.8	4.6	5.3	5.1	4.9	4.2	4.7	4.3	4.9
Spaulding Rose	8.3	6.9	8.4	6.0	5.3	7.1	7.8	6.5	7.4
Green Mountain	10.2	7.1	10.1	7.4	6.9	7.8	9.4	7.1	10.7
Bliss Triumph	14.3	9.7	11.4	8.4	9.8	10.7	7.8	7.5	7.8

the values from 10° and 15° being less important since storage at these temperatures cannot be carried out on account of excess sprouting.

Transferring from 5° to 27° for 16 days brought the reducing sugars to 5.0 mg. or lower (per cc. of juice) for all of the varieties tested except Bliss Triumph and possibly Green Mountain. When the transfer was from 5° C. to room temperature (22° C.), Russet Rural, Carman No. 3, Irish Cobbler, Chippewa, and Katahdin were well under the 5.0 mg. limit set for comparison; on transfer from 5° to 15° for 16 days Russet Rural and Chippewa were quite low in sugar; Carman No. 3 and Irish Cobbler, however, were approaching the 5.0 limit.

Barmore (2) mentions 15.6° C. (60° F.) as a suitable temperature at which to store the tubers after transfer from low temperature but this re-

fers to a six-week period of storage after transfer. For a period as short as 16 days it appears that the transfer should be to a higher temperature, at least to room temperature, or even  $27^{\circ}\text{C}$ .

There may be an objection to a storage temperature as high as  $27^{\circ}\text{C}$ . ( $81^{\circ}\text{F}$ .), since at this temperature desiccation of the tubers occurred and firmness of tubers was found to be important for the proper slicing of tubers in obtaining chips.

*Re-transfer from a high to a low temperature.* Tubers that had been stored for 16 days at temperatures of  $15^{\circ}$ , room temperature, and  $27^{\circ}$  were put in cold storage again, this time at  $5^{\circ}$  only, no lots being returned to either  $10^{\circ}$  or  $15^{\circ}$ . The object was to note whether the tubers of any of the varieties would develop reducing sugar sufficiently slowly, so that a second period at low temperature could be used to retard sprouting without undue increase in sugar. Samples of tubers were removed at intervals of 10 days, 3 weeks, and 6 weeks.

Table IV shows the reducing sugar content at the end of three weeks' storage at  $5^{\circ}\text{C}$ . of tubers that had been held for 16 days previously at higher temperatures. The values in Column 2, Table IV, show that if the original storage temperature was  $5^{\circ}$ , only the tubers of the varieties Russet Rural, Chippewa, and possibly Carman No. 3 could be placed at  $15^{\circ}$  for 16 days and then at  $5^{\circ}$  for 3 weeks and still have a sufficiently low reducing sugar value for satisfactory chip color. If, however, the intermediate 16-day period was at room temperature or  $27^{\circ}\text{C}$ . (Columns 3 and 4), not only these three varieties but in addition Irish Cobbler and Katahdin could be returned to cold storage at  $5^{\circ}$  for as much as 3 weeks without an increase in reducing sugar to such an extent as to make the tubers unsuitable for chips. All of the other varieties from the *original* storage at  $5^{\circ}\text{C}$ . for 62 days, however, had increased in the sugar content of the tubers to such an extent that the chips made from them were too dark in color.

Data showing the situation at the end of the 6-week storage period at  $5^{\circ}\text{C}$ . after the return of tubers to cold storage following a temporary period at higher temperatures are not given in detail in this paper. In general, tubers of Russet Rural were found still to be low in reducing sugar and to furnish chips of good color; tubers of Chippewa were satisfactory except when the intermediate temperature was at  $27^{\circ}\text{C}$ .; and those of the other varieties were unsuitable for chips under this condition.

#### SUMMARY

Tubers of 11 varieties of potatoes of the 1939 harvest were placed in storage on December 1, 1939 at different cold storage temperatures. At subsequent periods samples were transferred to higher temperatures, and later samples of these were again placed in cold storage. At intervals samples of tubers were removed from the various lots, the sugar contents of

the juices were determined, and potato chips were prepared from the tubers.

The critical factor which determined the color of the potato chips was not the total sugar content of the tuber, as had been assumed previously, but only the content of reducing sugar. Good correlation was found between the amount of reducing sugar in the juice and the extent of browning when chips were prepared from the tubers which furnished the juice, but there was no correlation between sucrose content and color of chips.

Experiments with pieces of filter paper which had been soaked in sugar solutions and starch suspensions, and which were then cooked in fat in a manner similar to that used with potato chips, confirmed these conclusions.

Large differences were found in the reducing sugar contents (and consequently in chip color) of the varieties used, not only at the start of the tests but also after various storage periods at different temperatures. Tubers of the variety Russet Rural were consistently low in reducing sugar (but not in sucrose) and furnished chips very light in color, too light in most cases, so that to obtain chips sufficiently brown the temperature of the cooking fat had to be increased. Other varieties which were rather low in reducing sugar and which produced chips of good color under most of the conditions were Chippewa, Carman No. 3, Irish Cobbler, and Katahdin. Varieties consistently high in reducing sugar and which gave chips too brown in color in most of the tests were Bliss Triumph and Green Mountain. The varieties Russet Burbank, Blue Victor, Early Ohio, and Spaulding Rose were intermediate with regard to both reducing sugar and color of potato chips.

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# HYBRIDS RESULTING FROM CONTROLLED POLLINATION OF *LILIUM SULPHUREUM* WITH *L. HENRYI* POLLEN

NORMA E. PFEIFFER

In June, 1940, three fine hybrid plants (Figs. 1 and 2) from seed produced by controlled pollination of *Lilium sulphureum* with pollen of Henry's lily flowered in the greenhouse at Boyce Thompson Institute. The two lilies giving rise to these hybrids are known scientifically as *Lilium myriophyllum* var. *superbum* (Baker) E. H. Wilson (syn. *L. sulphureum* Baker) and *L. henryi* Baker.

The characters of the seedlings vary, but all show definitely the effect of a combination of the two parents. In general, the vegetative characters resemble those of the seed parent although no two seedlings have equally narrow leaves or the same height of stem or same degree of greenness (there is more or less dark reddish flecking of the stem, as in *L. henryi*).

The flowers of all three are intermediate in form and coloring between the white funnel with sulphur yellow throat of the seed parent and the orange of the recurved perianth of the nodding flowers of the pollen parent. Flowers of seedlings 1 and 2 resemble each other more than either resembles seedling 3. The flower of the third seedling is larger and has more nearly uniform coloring than the others. The large, widely expanded flowers and the coloring in seedlings 1 and 2 agree in general with the published description (4) of *Lilium* T. A. Havemeyer; the leaves, however, are not of the "small, broadly lanceolate type" mentioned there. A description of this same hybrid in the Lily Year-Book of the Royal Horticultural Society indicates "foliage after form of *L. henryi*" (5, p. 2) and "spreading linear-lanceolate leaves" (3).

In the same year, Dr. A. B. Stout (7) gave a more detailed description of this hybrid and discussed its probable origin from *L. sulphureum* with *L. henryi* pollen. However, in the opinion of the producer of *Lilium* T. A. Havemeyer, Mr. Tom Barry, *L. tigrinum* pollen had been responsible for seed production on *L. sulphureum* in his original cross (1), although he indicated that *L. henryi* characteristics entered in.

The present results of a controlled pollination producing seedlings with similar flowers to those of *Lilium* T. A. Havemeyer seem to leave no doubt that the cross was between *L. sulphureum* and *L. henryi*, as Stout (7) and Stern (2, p. 45; 6, p. 84) have concluded.

Further detailed description and illustrations of the three seedlings will appear in a later paper.

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FIGURE 1. Seedling 1, side and face views; hybrid produced by pollination of *Lilium sulphureum* with *L. henryi* pollen. About one-sixth natural size. June 13, 1940.



FIGURE 2. Seedlings 2 and 3; resulting from same pollination. Basal portions of plants are omitted. June 13, 1940.

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# AN ANALYSIS OF FACTORS CAUSING VARIATION IN SPORE GERMINATION TESTS OF FUNGICIDES. II. METHODS OF SPRAYING<sup>1</sup>

S. E. A. McCallan and Frank Wilcoxon

The variations in spore germination tests of fungicides arise from two sources—biological and mechanical. In the first paper of this series (6) the biological errors associated with the methods of isolating, producing, and germinating the spores were studied. The present paper compares the various techniques of spraying and analyzes the magnitude of the mechanical errors. A new method capable of reasonably high precision is developed.

## METHODS OF SPRAYING

In toxicity tests on higher animals it is usually true that the dose applied is quite accurately known, and the theoretical discussions on toxicity curves usually take this for granted. In the laboratory testing of fungicides, on the other hand, it is a matter of considerable difficulty to apply a known amount of fungicide to glass slides in a reproducible manner.

There are three basic methods of applying sprays: freehand spraying, stationary horizontal sprayers, and settling towers. Various modifications of the two former methods have been used extensively for applying fungicides while the settling tower technique is presented here for the first time. An entirely different procedure employing a micro-pipette has been introduced by Montgomery and Moore (9). This method was not studied because it is not a method of spraying and because it does not avoid the error of settling of deposit while pipetting. The primary object in the present paper has been to compare the reproducibility of slides sprayed at the same and at different times by the various methods.

## FREEHAND SPRAYING

In the original method of Reddick and Wallace (10) the sprays were applied by means of a bulb atomizer. This method has been used widely with attempts to regulate the deposit by counting the number of "squirts" or by visual inspection. In testing this method a deVilbiss No. 15 atomizer was employed (the same nozzle was used in all subsequent methods), and different amounts of spray applied by giving 15, 30, 45, or 60 "squirts" to a row of four glass slides. The spray was applied from three sides while the operator walked in a semicircle. The nozzle was held about 6 to 8 inches

<sup>1</sup> A preliminary report on this paper was presented before the American Phytopathological Society, Columbus, Ohio, December, 1939 (7).

above and 20 inches away from the slides. It was impossible to get an even deposit of spray because of frequent blobs of spray.

*Freehand spraying with controlled pressure and time.* This modification is an attempt to give better regulation of the deposit. The procedure was that employed in an earlier article (5), namely the atomizer was attached to the laboratory air pressure line and the pressure regulated to 400 mm.<sup>2</sup> mercury by means of a manometer. The spray was applied to a row of four slides for 12 seconds, the slides being more or less evenly covered by passing the nozzle back and forth across them and applying from three sides as above. Different amounts of deposit were attained by making up different stocks or by dilution of one stock. During the spraying, an effort was made to keep the spray in the bottle agitated by shaking.

#### STATIONARY HORIZONTAL SPRAYERS

Various types of stationary horizontal sprayers have been developed. A number of these have been modified from a type developed by Evans and Martin (3). Doctors Horsfall, Heuberger, Sharvelle, and Hamilton (4)

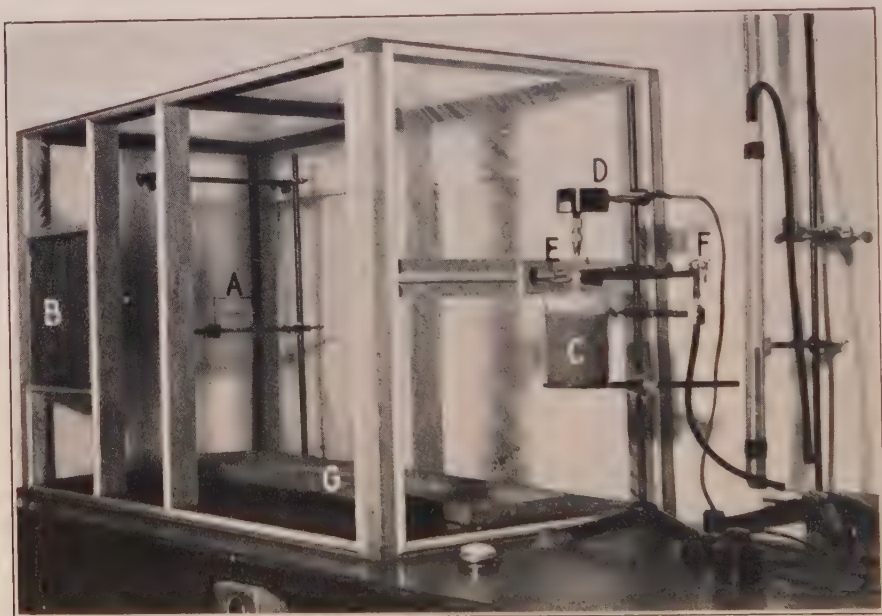


FIGURE 1. Stationary horizontal sprayer. A. Single slide mounted in horizontal position at far end of chamber; B. door for removing slide. Arrangement of ringstand, with C. beaker of spray suspension; D. stirrer; E. nozzle inserted through opening in chamber; and F. cut-off. G. Pan to catch excess spray.

<sup>2</sup> Not 40 mm. as stated in a typographical error in the original (5).

have experimented extensively with this type of sprayer, introducing a number of improvements, and we are indebted to them for many ideas.

The chief advantage of these sprayers, in addition to constant pressure, distance, and time, is that only one stock suspension of spray is prepared and kept constantly stirred so that no error results from dilution and sampling. Only one slide is sprayed at a time and the amount of deposit is regulated by the time of spraying.

Several different horizontal spray chambers were constructed and the most satisfactory regarding reproducibility of deposit, ease of construction and of operation is illustrated in Figure 1. A wooden framework  $22 \times 26 \times 44$  inches was made and covered with cellophane. The deVilbiss nozzle No. 15 is inserted through an inch hole at one end 14 inches above the table on which the whole apparatus is placed. The slide is held in a horizontal position 30 inches from the nozzle. This distance may be varied. The slide is removed through a small door on the side. Five hundred cc. of stock suspension are prepared and placed in a 600 cc. beaker which is firmly held on a ring stand. The nozzle and electric stirrer<sup>3</sup> are likewise attached to the ring stand which is secured to the table top. The timing of the spraying is regulated by a deVilbiss No. 631 cut-off. It is necessary to have a reducing valve inserted between the manometer and laboratory air supply where the latter is at a pressure substantially higher than that required for spraying. A pressure of 400 mm. was maintained, though the pressure, like the distance, may be varied with a resulting change in the amount of deposit. The time of spraying may be varied from about 4 to 40 seconds, depending on the surface tension of the material being applied and amount of deposit desired.

#### SETTLING TOWERS

The possibilities of employing a settling tower to give a uniform deposit of fungicidal spray do not appear to have been considered previously. The theoretical advantages of a settling tower are first, that air currents are greatly reduced, and secondly, that gravitational pull is constant, as compared to horizontal spraying where both of these factors may be a source of error. In addition, a large number of slides may be coated at the same time.

*Construction.* After experimenting with various designs a fairly simple and satisfactory settling tower was constructed and is shown in Figure 2. The basic dimensions are a foot square tower, five feet tall, with nozzle opening one foot from the bottom. The tower was constructed in two removable parts, upper of galvanized sheet steel and lower wooden base. The upper end of the tower is closed with a hinged wooden door. The upper edges of the wooden base are grooved to receive the metal tower. The front of the wooden base consists of a glass window and removable wooden door.

<sup>3</sup> Variable speed electric non-sparking stirrer. Eimer & Amend, New York, N. Y.

When the door is removed two sliding sheet metal trays may be pulled out. The upper tray which constitutes the bottom of the settling chamber holds the glass slides. The lower tray which is equipped with sides catches the excess spray. The spraying apparatus consisting of a No. 15 deVilbiss nozzle with tip turned up at right angles, stirrer, cut-off, and 600 cc. beaker containing the spray suspension is the same as that used for the cellophane horizontal spraying chamber.

*Operation.* The steps in the operation of the tower are described below. The times and pressure specified may of course be varied. This phase will be discussed later.

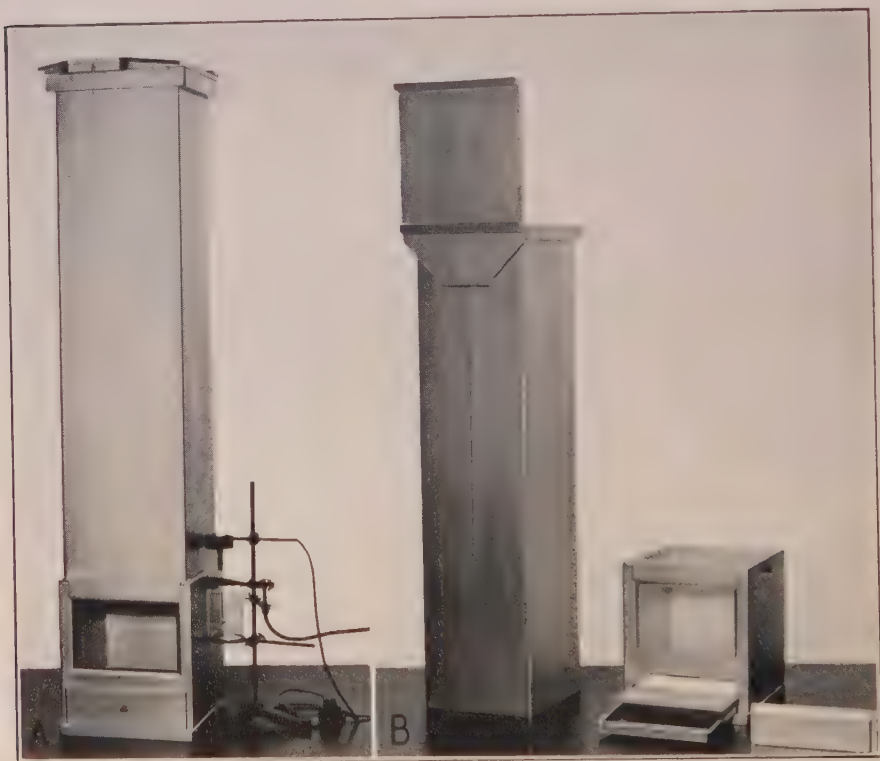


FIGURE 2. Simple "home-made" settling tower. A. Tower closed up and nozzle inserted for spraying. Same arrangement of ringstand as in Figure 1. B. Tower dismantled, showing upper and lower parts, top open, door removed, and trays partially removed. Upper tray holds slides.

1. Glass slides are placed on blotting paper on the slide tray, which is removed from the tower.

2. The tower is completely shut except for the nozzle opening through which the nozzle, with tip turned up, is inserted.



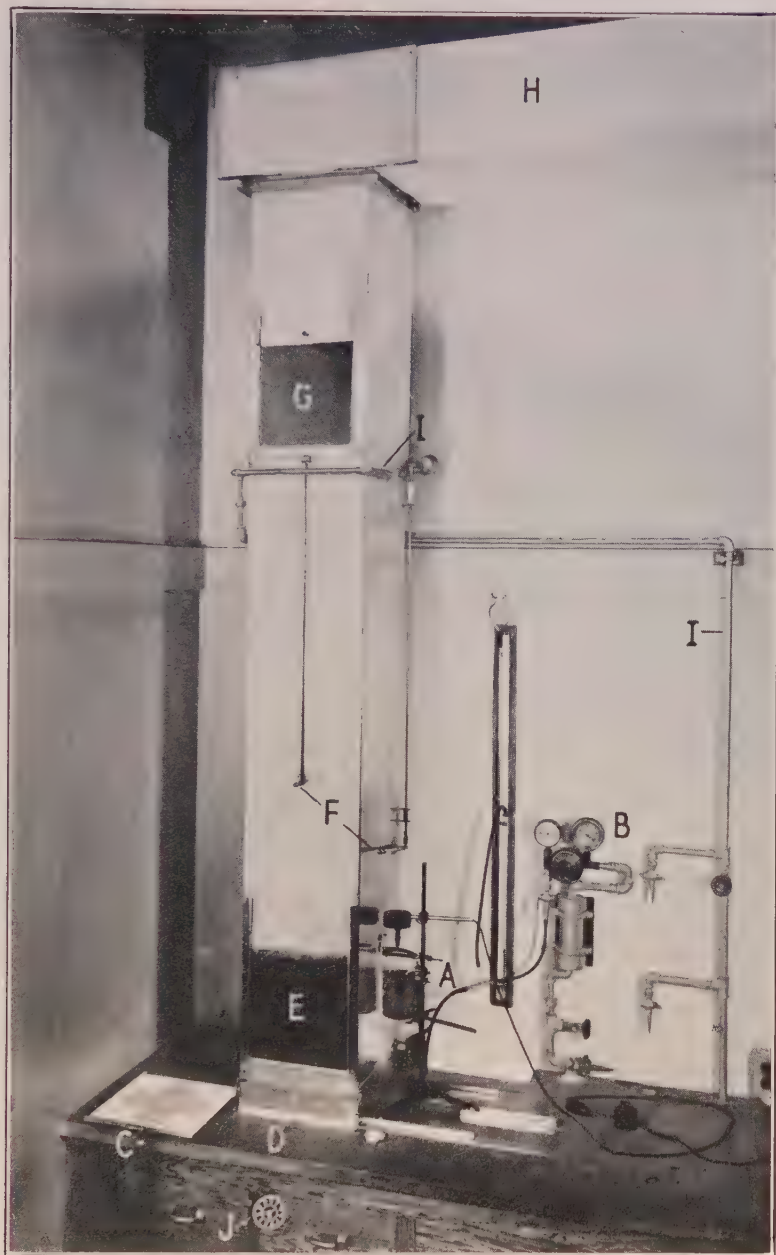


FIGURE 3. Permanently installed stainless steel settling tower. A. Ringstand apparatus on steel runway; B. reducing valve on air pressure line; C. tray of slides; D. automatic door through which tray is inserted; E. sliding glass window for draft; F. levers for opening and shutting top of settling chamber; G. upper window for adjustments on top, sprinkler nozzles, etc.; H. laboratory exhaust duct; I. water line to sprinkler nozzles for washing out; J. drain valve.

3. Spray for 30 seconds at 10 pounds' pressure (520 mm. Hg).
4. Withdraw nozzle and close opening with stopper.
5. Wait 10 seconds after end of spraying for preliminary settling of large drops, then open door and insert tray of slides. This operation must be done as quickly and carefully as possible.
6. After slides have been in the tower 60 seconds, withdraw the tray. Remove at random the number of slides needed.
7. Open top and by means of compressed air at bottom blow out residual fog of spray remaining in tower.

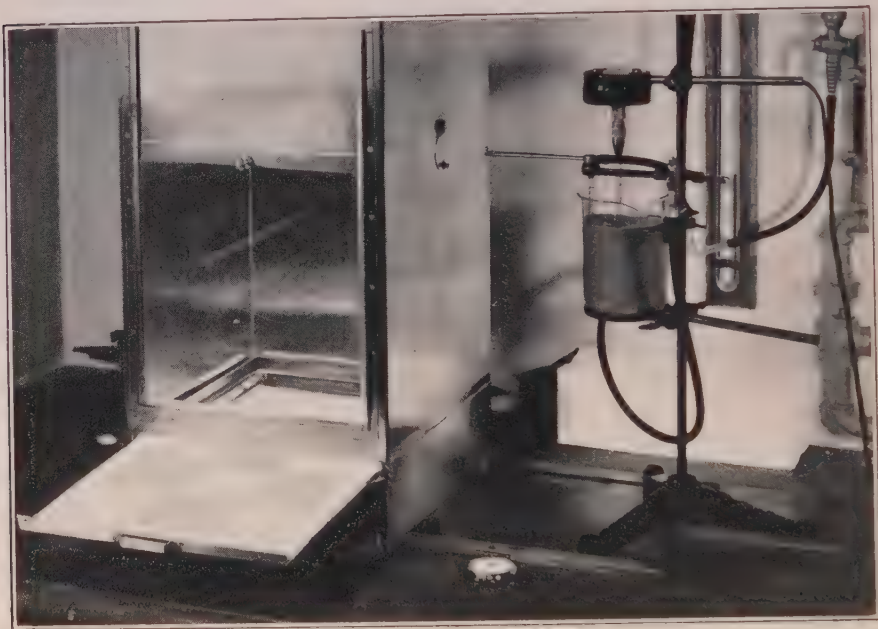


FIGURE 4. Close-up of stainless steel settling tower showing manner of inserting slide tray. Note "cross" arrangement of slides and steel runway for ringstand. Nozzle withdrawn from tower by sliding ringstand back, nozzle opening closed

8. For successive exposures close up tower and repeat operations. Varying amounts of spray deposit are obtained by varying the number of times a slide is exposed. No attempt is made to dry the slides between exposures unless excessively large amounts of deposit are required.

*Permanently installed stainless steel settling tower.* For greater convenience of operation a machine-made stainless steel tower was designed and installed. The precision of this tower was found to be the same as that of the simpler one just described. The stainless steel tower is illustrated in Figures 3 and 4. Its essential dimensions of a five foot tall tower, one foot square, remain as before. The features which permit faster and simpler

operation include automatic closing of tray door, forced draft for removing fog after exposure, sprinkler for washing out, drain at bottom, arrangement of slide tray grooves to permit escape of spray running down inside of tower, and stainless steel runners for sliding ringstand holding nozzle, etc., to and from tower.

#### COMPARISON OF METHODS BY MEANS OF DYE TESTS

Since this study is primarily a test of the mechanical reproducibility of spray deposits, it was considered desirable in the majority of tests not to complicate the results with biological variation. At first an attempt was made to spray with water and weigh the resulting deposit on the slide in a weighing bottle. This proved inaccurate and unsuitable for a number of slides sprayed at the same time and hence was discarded. Chemical analyses are also not suitable, since a very small amount of material is deposited on a single slide.

The use of a dye was found to be very satisfactory, giving rapid and

TABLE I  
TESTS OF FREEHAND SPRAYING WITH BULB ATOMIZER. MILLIGRAMS OF DYE  
SOLUTION DEPOSITED PER SLIDE. FOUR REPLICATE SLIDES

Dose No. "squirts" of 1.0% dye	Test				
	1	2	3	4	5
15	6.1	6.8	16.5	11.3	9.0
	6.4	6.1	21.6	14.6	12.2
	6.2	8.8	21.6	14.8	10.0
	10.9	10.7	22.1	12.8	11.4
30	15.9	29.0	17.0	30.5	23.0
	21.7	35.3	25.0	31.0	31.7
	17.1	32.1	24.4	32.7	38.9
	18.6	30.2	26.8	26.1	34.1
45	19.4	43.8	79.5	55.8	53.6
	37.7	51.4	72.3	52.2	62.8
	26.7	52.8	56.2	55.1	60.7
	30.0	53.6	59.7	56.4	63.2
60	56.3	73.2	98.4	79.7	101.7
	62.2	84.5	119.2	83.3	61.8
	61.5	73.8	94.8	86.9	81.1
	56.3	65.9	95.3	77.5	54.1

#### Analysis of Variance

	Degrees of freedom	Sums of squares	Variance	$\sigma$
Replicate slides	60	3001.06	50.02	7.07
Replicate tests	4	5221.85	1305.46	36.13
Doses	3	50981.98	16993.99	
Tests $\times$ doses	12	3090.53	257.54	
Total	79	62295.42		

TABLE II

TESTS OF FREEHAND SPRAYING WITH CONTROLLED PRESSURE (400 MM. Hg) AND TIME (12 SEC.), MILLIGRAMS OF DYE SOLUTION DEPOSITED PER SLIDE.  
FOUR REPLICATE SLIDES

Dose % dye in stock solution	Test				
	1	2	3	4	5
0.1	37.3	29.0	33.9	29.8	25.6
	43.0	31.7	36.8	31.2	27.4
	46.5	33.2	37.4	32.7	28.8
	42.9	30.3	34.9	30.4	30.4
0.2	31.8	40.9	37.0	34.6	33.1
	40.2	40.9	39.7	35.3	37.4
	40.2	40.3	42.2	37.0	35.1
	40.1	41.7	40.8	36.9	34.2
0.3	39.0	27.6	31.6	27.4	25.9
	39.4	30.7	32.3	29.6	29.2
	46.3	36.3	32.7	28.9	32.1
	44.7	31.7	30.5	28.2	34.2
0.4	35.1	26.3	30.8	27.3	32.2
	40.0	26.8	29.5	26.9	33.4
	41.8	28.8	30.4	29.7	35.7
	36.6	31.4	30.9	30.2	34.1

## Analysis of Variance

	Degrees of freedom	Sums of squares	Variance	$\sigma$
Replicate slides	60	345.51	5.76	2.40
Replicate tests	4	876.40	219.10	14.80
Doses	3	428.73	142.91	
Tests $\times$ doses	12	493.39	41.12	
Total	79	2144.03		

reasonably precise results. The dye selected was Brilliant Blue F. C. F.<sup>4</sup> and stock solutions of 1.0 per cent were usually sprayed. After spraying, the dye deposit from each slide was washed off and made up to 10 cc. and the amount determined in a colorimeter. The coefficient of variation for replicate colorimeter readings was about 1.3 per cent.

*Results.* A comparison of the four methods of spraying was made in which the variables were: (a) four replicate slides sprayed at same time (in succession for the single slide horizontal sprayer), (b) four different doses, i.e., stock solutions, times or exposures, and (c) five replicate tests on different days. The detailed results on the four methods together with their analyses are given in Tables I to IV.

In order that the results may be compared directly, the coefficients of variation for replicate slides (slides sprayed at same time) and replicate

<sup>4</sup> Coal tar dye. National Aniline & Chemicals Co., Inc., New York, N. Y.



TABLE III

TESTS OF STATIONARY HORIZONTAL SPRAYER. MILLIGRAMS OF DYE SOLUTION  
DEPOSITED PER SLIDE, FOUR REPLICATE SLIDES

Dose 1.0% dye; settings for time and pressure	Test				
	1	2	3	4	5
5 sec. 400 mm.	17.8	24.9	21.7	22.1	19.3
	21.9	23.6	22.1	20.9	19.4
	18.7	21.1	22.5	21.0	19.2
	20.2	25.3	23.3	21.4	19.4
10 sec. 400 mm.	37.5	44.2	45.8	37.3	35.3
	43.7	44.5	41.9	38.6	38.5
	43.3	42.6	43.5	39.2	36.6
	43.2	46.5	40.8	40.9	34.7
5 sec. 500 mm.	27.4	29.3	26.0	27.4	24.4
	27.8	31.6	27.8	25.7	24.2
	27.1	33.3	31.9	30.3	23.2
	28.1	31.6	28.7	23.3	22.5
10 sec. 500 mm.	52.8	62.5	55.8	51.1	47.4
	50.2	54.3	50.7	52.6	48.9
	48.1	54.3	49.6	55.6	44.2
	52.8	51.9	52.6	51.9	47.4

## Analysis of Variance

	Degrees of freedom	Sums of squares	Variance	$\sigma$
Replicate slides	60	257.00	4.28	2.07
Replicate tests	4	454.61	113.65	10.66
Doses	3	11153.13	3717.71	
Tests $\times$ doses	12	66.73	5.56	
Total	79	11931.47		

tests (slides sprayed at different times) have been calculated and are presented in Columns 2 and 3 of Table V. In addition, the variance of slides within doses is given in Column 4. The differences between all the methods is highly significant. Thus by introducing constant pressure and time to the freehand technique an increase in precision of nearly seven-fold resulted. By changing to the horizontal sprayer a further two-fold increase in precision resulted. Finally, the settling tower brought about another two-fold increase over the horizontal sprayer or greater than thirty-fold over the original method.

## CALIBRATION OF SETTLING TOWER

Factors known to regulate the spray deposit in the settling tower are: time of spraying, time of preliminary settling, time of exposure, spray pressure, and position of slide. The results of varying each of these factors with all others held constant are shown in Figure 5.

TABLE IV

TESTS OF SETTLING TOWER. MILLIGRAMS OF DYE SOLUTION DEPOSITED PER SLIDE.  
FOUR REPLICATE SLIDES

Dose No. exposures to 1.0% dye	Test				
	1	2	3	4	5
1	15.0	14.9	16.2	16.0	15.2
	13.7	15.0	15.9	16.8	16.5
	14.7	16.8	17.0	15.3	17.3
	14.4	15.4	15.7	16.4	17.9
2	30.6	31.4	31.3	30.6	30.2
	26.8	28.6	30.0	31.9	32.8
	31.1	32.3	31.8	31.6	33.3
	26.4	30.3	28.9	32.3	31.6
3	46.5	48.3	44.8	47.8	45.0
	43.3	44.0	48.1	47.8	47.7
	48.8	47.8	48.3	48.1	46.4
	42.7	48.8	44.4	49.4	48.6
4	57.7	62.5	59.8	63.3	59.8
	54.1	58.2	62.5	62.5	65.8
	60.3	63.5	61.1	59.7	63.5
	57.2	58.0	61.1	63.8	65.2

## Analysis of Variance

	Degrees of freedom	Sums of squares	Variance	$\sigma$
Replicate slides	60	183.18	3.05	1.75
Replicate tests	4	112.04	28.01	5.29
Doses	3	23030.39	7676.80	
Tests $\times$ doses	12	33.67	2.81	
Total	79	23359.28		

TABLE V

COMPARISON OF VARIATION OF SPRAY DEPOSIT ON REPLICATE  
SLIDES BY DIFFERENT METHODS OF SPRAYING

Method of spraying	Coefficient of variation for slides sprayed at		Variance of slides within doses. 76 degrees freedom
	Same time	Different times	
Freehand with bulb atomizer	16.7	85.2	148.86
Freehand with controlled pressure and time	7.0	43.4	22.57
Stationary horizontal sprayer	5.9	30.1	10.25
Settling tower	4.5	13.7	4.33

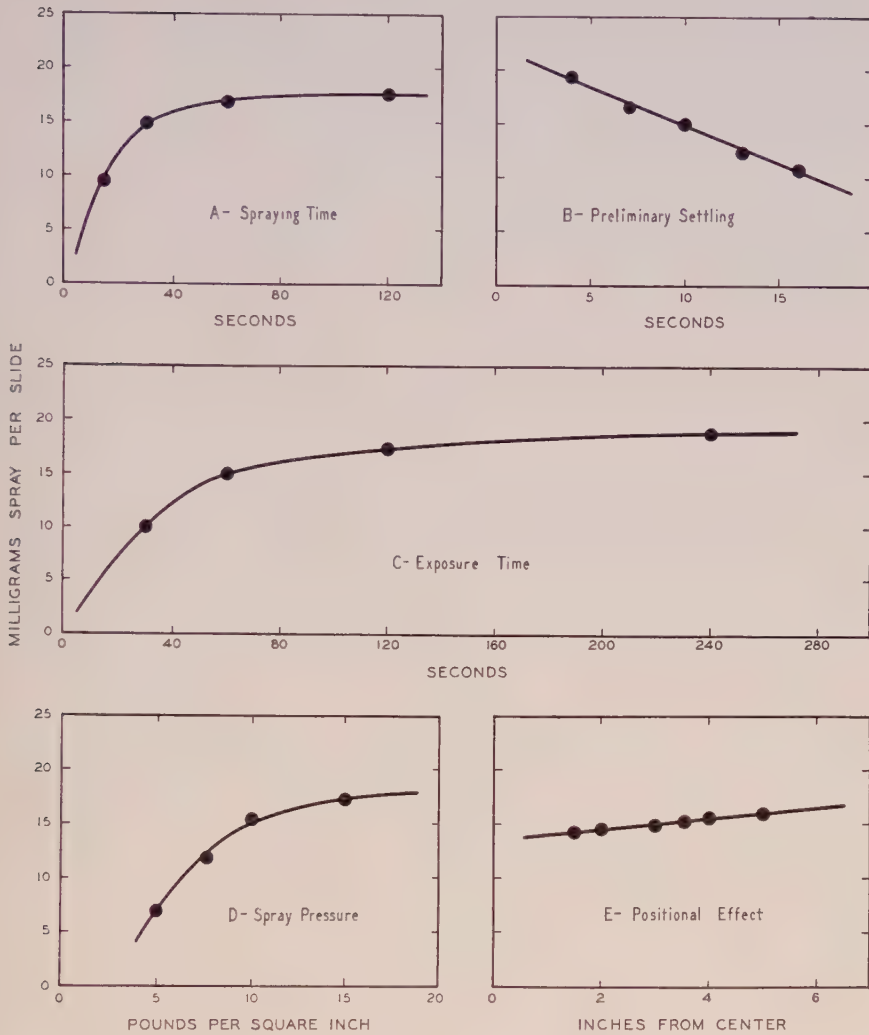


FIGURE 5. Calibration curves for settling tower. Milligrams of spray deposited per slide ( $3'' \times 1''$ ) for the different variables of A. spraying time; B. preliminary settling time; C. exposure time; D. spraying pressure; and E. positional effect in tower. While studying each variable, others held constant as follows: A. 30 sec., B. 10 sec., C. 60 sec., D. 10 lbs., and E. average of all positions.

The final values of 30 seconds' spraying, 10 seconds' settling, 60 seconds' exposure, and 10 pounds' pressure were selected as most desirable since they permit reasonable speed of operation while maintaining high precision.

*Positional effect.* In the results reported in Table IV, the slides were chosen at random from each of the four quarters and no consistent quarter ef-

fect could be demonstrated. However, when the slides were arranged in a number of smaller areas a concentric gradient was noted with a smaller deposit in the center of the tower as is shown in Figure 5 E. This positional effect can be allowed for by a simple arrangement of slides in the form of a cross with three slides to each arm (see Fig. 4). With the settings stated above, the inner slides two inches from the center will receive approximately 14.5 mg. of spray per slide ( $3 \times 1$  inch) or 0.750 mg. per sq. cm., the next row of slides three inches from the center 15.0 mg. per slide or 0.775 mg. per sq. cm., and the third row at four inches, 15.5 mg. per slide or 0.800 mg. per sq. cm. More slides may be exposed by replacing the ones removed with fresh ones during the operation.

*Different nozzles.* Eight different deVilbiss No. 15 nozzles of apparently the same design and obtained at different times were compared in five positions and two different tests. No significant difference could be shown for different nozzles compared to the same nozzle in different tests.

*Dose ratios.* Varying dose ratios may be attained by combinations of different stock suspensions and number of exposures. For example, dose ratios of 2 and 3 may be had by exposing respectively 1, 2, 4, and 8 times, and 1, 3, and 9 times. By preparing two stocks, the first of 1.0 concentration units and the second of 1.414, and applying each for 1, 2, and 4 exposures, six doses with a ratio of 1.414 will result.

#### EXPECTED AND OBSERVED DEPOSITS WITH COPPER FUNGICIDES

It may be argued that results with a dye solution are not necessarily comparable to insoluble spray materials such as copper fungicides. Accordingly, four fungicides—Standard Laboratory Bordeaux (1), and three commercial materials, consisting of a cuprous oxide, a basic sulphate, and an hydroxide—were applied in the settling tower. The difference in per cent copper in the stock suspension ranged from 0.02 to 0.10 per cent for the different materials. Each material was applied to twelve slides for eight exposures.

The spray deposit was dissolved off the slides with 2 per cent nitric acid, the acid evaporated on a steam bath, and the residue taken up with concentrated sulphuric acid. Copper determinations were made in a colorimeter by means of the sodium diethyl dithiocarbamate test (2).

The results of the amount of deposit expected from calibration by the dye method, and of the amount observed in the chemical test are given in Table VI. It will be seen that the differences are both positive and negative and that the mean difference of 5.4 per cent is reasonable in comparison with the errors of the colorimetric chemical determination. Thus it is concluded that the dye calibration will give a satisfactory value for the expected deposit of an insoluble fungicide. Fungicides of unusual surface tension or weight, however, may require a special calibration.



TABLE VI

COMPARISON OF EXPECTED DEPOSIT FROM DYE CALIBRATION OF SETTLING TOWER, WITH OBSERVED DEPOSIT BY SODIUM DIETHYL DITHIOCARBAMATE COLORIMETER TEST. MILLIGRAMS COPPER DEPOSITED ON TOTAL OF 12 SLIDES AND 8 EXPOSURES

Fungicide	Expected	Observed	Per cent difference
Standard Laboratory Bordeaux	0.288	0.307	6.2
Commercial basic sulphate	0.360	0.329	9.4
Commercial copper hydroxide	0.360	0.370	2.7
Commercial cuprous oxide	1.440	1.490	3.4

## COMPARISONS BY SPORE TESTS

Since spore germination tests of fungicides are a method of bioassay involving a quantal response (11), we may make use of the criteria of Miller, Bliss, and Braun (8) for choosing between alternative techniques.

These authors specify four criteria for evaluating precision, namely (a) low  $\chi^2$  values for linearity of the dose-effect curve; (b) high values for slope  $b$ ; (c) low values for standard error of the log-ratio of potencies; (d) reproducibility.

Data have been collected from time to time on these methods of spraying, in which the effects of different copper fungicides have been observed on different fungi. These data have been examined to determine how the methods compare in their response to these criteria. No comparison on (c) has been made since in spore germination tests a standard is frequently not employed.

*Linearity of curves.* Graphic calculations of  $\chi^2$  values by the method previously described (11) were obtained from comparable tests for Standard Laboratory Bordeaux and a commercial cuprous oxide on the spores of *Sclerotinia fructicola* (Wint.) Rehm., *Botrytis* sp. (*cinerea* type), *Alternaria solani* (Ell. & Mart.) Jones & Grout, and *Macrosporium sarcinaeforme* Cav. and appear in Table VII. It will be seen that the  $\chi^2/n$  values are lowest for the settling tower, and next lowest for the stationary horizontal sprayer.

TABLE VII

$\chi^2$  TESTS ON LINEARITY OF DOSE-EFFECT CURVES BY THREE METHODS OF SPRAYING FOR STANDARD BORDEAUX AND CUPROUS OXIDE ON SPORES OF *SCLEROTINIA FRUCTICOLA*, *BOTRYTIS CINEREA*, *ALTERNARIA SOLANI*, AND *MACROSPORIUM SARCINAEFORME*

Method of spraying	$\chi^2$	$n$	$\chi^2/n$
Freehand with controlled pressure and time	563.9	68	8.29
Stationary horizontal sprayer	596.0	122	4.89
Settling tower	231.5	74	3.13

*Steepness of slope.* Seven different commercial copper fungicides were tested against *Sclerotinia fructicola* in eight replicated tests, for the free-

hand spraying with controlled pressure and time and for the settling tower. The slopes were obtained graphically and the mean slopes and variances are given in Table VIII. These were compared for significance by the *t* test and in the majority of compounds the settling tower gave a significantly greater slope. The lower slope of the freehand method is perhaps due to errors of settling which result in less deposit than expected at the higher concentrations.

TABLE VIII  
COMPARISON OF STEEPNESS OF SLOPES (*b*) OF DOSE-EFFECT CURVES FOR TWO METHODS OF SPRAYING. RESULTS WITH 8 REPLICATED TESTS ON *SCLEROTINIA FRUCTICOLA*

Type of commercial copper fungicide	Freehand spraying controlled pressure and time		Settling tower		Difference in favor of settling tower
	Mean	Variance	Mean	Variance	
Cuprous oxide	0.404	0.00945	0.660	0.00486	Highly significant
Phosphate	0.207	0.00416	0.332	0.03780	Highly significant
Zealite	0.459	0.01450	0.667	0.00210	Highly significant
Basic sulphate	0.396	0.00989	0.631	0.00667	Highly significant
Silicate	0.391	0.02900	0.563	0.00497	Significant
Oxychloride	0.545	0.00393	0.575	0.00637	Not significant
Hydroxide	0.683	0.00769	0.544	0.07440	Significant in favor of freehand spraying

*Reproducibility.* Two commercial copper fungicides were tested against *S. fructicola* on four different days. On each day both methods of spraying were completely replicated and the resulting slides tested with the same lot of spores. By using the same lot of spores, the day-to-day variability of different lots of spores (6) was not introduced. A  $\chi^2$  test was made on the replicate sprayings within a given dose, compound, day, and method of spraying. The totaled results are given in Table IX. The settling tower technique is seen to be more reproducible.

TABLE IX  
 $\chi^2$  TEST FOR REPRODUCIBILITY OF REPLICATED SPRAYINGS USING THE SAME LOT OF SPORES. DATA ON PERCENTAGE GERMINATION OF *SCLEROTINIA FRUCTICOLA* WITH TWO COMMERCIAL COPPER FUNGICIDES

Method of spraying	$\chi^2$	<i>n</i>	$\chi^2/n$
Freehand, controlled pressure and time	528.8	38	13.92
Settling tower	118.7	20	5.94

### SUMMARY

I. A comparison was made of the relative precision of four methods of spraying fungicides in the laboratory. The four methods were (a) freehand spraying with bulb atomizer, (b) freehand spraying with controlled pres-

sure and time, (c) stationary horizontal sprayers, and (d) settling towers.

2. The most satisfactory type of horizontal sprayer was a cellophane-covered chamber with the glass slide mounted about 30 inches from an atomizer nozzle. The spray suspension was stirred continuously, applied at constant pressure, and amount of deposit regulated by varying time of spraying.

3. A simple "home-made" settling tower and a more elaborate stainless steel one were developed, the latter being easier to operate. Both were basically one foot square, five foot tall towers. The spray is directed up into the tower, discontinued, and after an interval, slides are introduced which receive a uniform coating of fine spray. The amount of deposit is varied by building up with successive exposures.

4. The four methods were compared by dye tests, in which Brilliant Blue dye was sprayed, then dissolved off the slides and the amount determined in a colorimeter. Each method was tested at five different times, for four doses and four replicate slides. The coefficients of variation for slides sprayed at the same and at different times were respectively for the four methods: (a) 16.7 and 85.2 per cent, (b) 7.0 and 43.4, (c) 5.9 and 30.1, and (d) 4.5 and 13.7, while the variances for replicate slides within doses, 76 degrees freedom, were (a) 148.86, (b) 22.57, (c) 10.25, and (d) 4.33. These variances are all significantly different.

5. A comparison of observed deposit of various copper fungicides in the settling tower with that expected by calibration with the dye showed an average agreement within 5.4 per cent.

6. Comparisons of the methods by actual spore germination tests as regards  $\chi^2$  test for linearity of dose-effect curve, steepness of slope, and reproducibility of replicates indicated the settling tower to be the most precise.

7. It is concluded directly from dye and indirectly from spore germination tests that the settling tower method of applying sprays is the most precise, followed in order by stationary horizontal sprayers, freehand spraying with controlled pressure and time, and last, freehand spraying with bulb atomizer.

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# TOXICITY OF AMMONIA, CHLORINE, HYDROGEN CYANIDE, HYDROGEN SULPHIDE, AND SULPHUR DIOXIDE GASES. I. GENERAL METHODS AND CORRELATIONS

S. E. A. McCallan and Carl Setterstrom

A series of cooperative tests were conducted in which a survey was made of the relative toxicity of five common industrial gases to various forms of plant and animal life. The apparatus employed permitted a continuous flow of the gas at definite concentrations. It is believed that this is the first survey attempted under such conditions.

The gases studied—ammonia, chlorine, hydrogen cyanide, hydrogen sulphide, and sulphur dioxide—were selected because of their chemical properties, physiological action, and their occurrence in industrial atmospheres. The organisms were chosen to give a wide range of behavior in the hope that a broader picture of the mechanism of toxic action might result. A total of 18 different species were studied, including plant and animal pathogenic fungi and bacteria, green plants, seeds, insects, and rodents. In some cases a given species was studied under different conditions, or in different forms or organs.

This first paper of the series will describe the general methods employed and present a summary correlation of the results on all organisms. Succeeding papers (5, 7, 1, 9) will describe the original data in detail for the various organisms as follows: II. Fungi and bacteria, III. Green plants, IV. Seeds, and V. Animals.

## APPARATUS

The apparatus, employed in previous studies on sulphur dioxide, has been described in detail by Setterstrom and Zimmerman (6). It consists of duplicate sets of equipment housed in a small greenhouse which is provided with partial automatic temperature control. There are included a cabinet in which the living material is placed during the experiment; a variable speed exhaustor blower and an orifice meter for precise control of the volume of air passing into the cabinet; scrubbers attached to the intake of the blowers to rid the air of  $\text{SO}_2$  and other impurities before allowing it to enter the system, and to effect a partial control of the temperature and humidity in the cabinet; and a Thomas autometer or sampling mechanism and a conductivity recorder. The gases used were obtained from commercial cylinders and were metered through calibrated capillary flowmeters which were checked at intervals with the autometer-recording system. When HCN was used, the liquid from the commercial cylinder was passed through a coil heated by water and steam, through a diaphragm

reducing valve, and through a liquid trap before metering. In order to prevent HCN condensation the apparatus was kept at a temperature of approximately 80° F.

#### EXPERIMENTAL DESIGN

The organisms were exposed to different concentrations of the gases for different lengths of time, removed to a favorable environment and the per cent dead or alive noted after a given time. For the various classes of organisms specific observations were made on symptoms, pH changes, delay in germination, and other responses. In order to obtain as comprehensive information as possible, a wide range of concentrations and time periods was employed. Since Gaddum (4) and others (2, 8, 10) have demonstrated that the distribution of lethal doses tends to be symmetrical when plotted against the logarithm of the dose, the increments of concentration and time were varied in geometric ratio. Accordingly, each gas was studied at a concentration of 1, 4, 16, 63, 250, and 1000 parts per million parts of air by volume, and for time periods of 1, 4, 15, 60, 240, and 960 minutes. Both concentration and time intervals will be seen to provide a dose ratio of approximately four. This ratio is considered adequate for a survey of this type but for a more precise study of the toxicity curves lower dose ratios would be necessary.

The experiments were performed during the months of September, October, November, and December, and in order to minimize temperature and light intensity fluctuations the tests were begun at 5:30 p.m., the 16-hour period thus ending at 9:30 a.m. the following morning. Temperature and relative humidity readings were taken at hourly intervals. For the entire series of tests, exclusive of the HCN runs, the mean temperature was 73.3° F. with a standard deviation of 3.3, while the mean relative humidity was  $74.6 \pm 8.8$  per cent. In the case of the hydrogen cyanide the temperature was  $79.6 \pm 3.5^\circ$  F., and the relative humidity  $73.8 \pm 6.7$  per cent.

#### GENERAL CORRELATION OF RESULTS

A general correlation of the toxicity of various gases to the various organisms as obtained by the different investigators has been attempted. It now has become well established that the most precise point at which to compare toxicity is at the point of 50 per cent kill (8). Usually the concentration or dose killing 50 per cent at a constant time is reported, that is the LD<sub>50</sub> (8). However, in these studies the actual time of death for the animals in the various concentrations of gas was recorded, and it is thus preferable to specify the time to kill 50 per cent at a constant concentration. This unit will be designated the LT<sub>50</sub>. A constant concentration of 1000 p.p.m. was chosen because of the lack of toxicity of the lower concentration in many cases. It should be noted that due to differences in the slope of

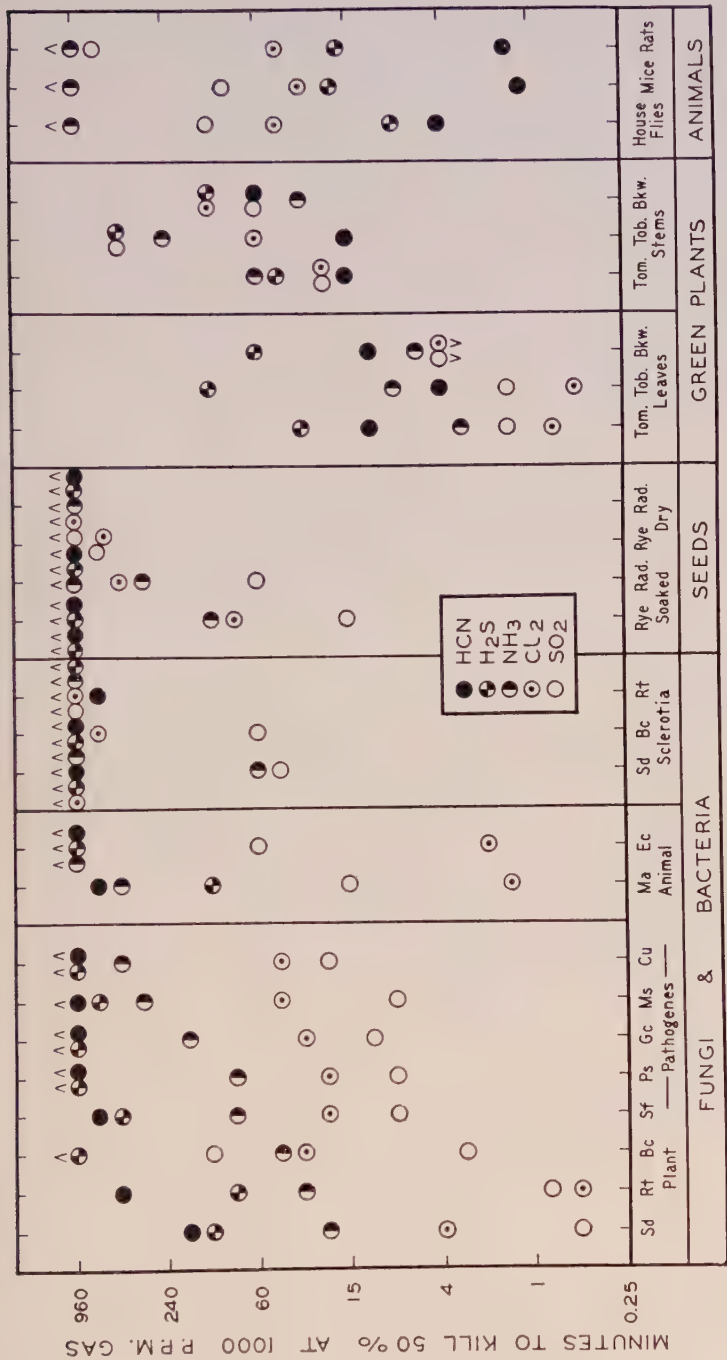


FIGURE 1. Comparison of LT<sub>50</sub> values in minutes at 1000 p.p.m. for different gases and organisms. Abbreviations of organism names from left to right as follows: Plant pathogene cultures—*Sclerotium delphinii*, *Rhizoctonia tuliparum*, *Botrytis* sp. (*cinerea* type), *Sclerotinia fructicola*, *Pestalotia stellata*, *Glomerella cingulata*, *Macrosporium sarcinaeforme*, and *Ceratostomella ulmi*; animal pathogene cultures—*Monilia albicans* and *Escherichia coli*; plant pathogene sclerotia—*Sclerotium delphinii*, *Botrytis* sp. (*cinerea* type), and *Rhizoctonia tuliparum*; seeds—soaked rye and radish, dry rye and radish; green leaves—tomato, tobacco (*Nicotiana glutinosa*), and buckwheat; green stems—ditto; animals—house flies, mice, and rats. Symbol  $\wedge$  indicates greater than, and  $\vee$  less than.

TABLE I  
RELATIVE TOXICITY OF GASES AND SENSITIVITY OF ORGANISMS. SIGNIFICANT DIFFERENCE WITH ODDS OF 20:1 OR GREATER,  
BETWEEN EACH GROUP

A. Order of Toxicity of Gases to Classes of Organisms				
Fungi and Bacteria	Seeds and Sclerotia	Green Leaves	Green Stems	Animals
1. SO <sub>2</sub> , Cl <sub>2</sub> 2. NH <sub>3</sub> 3. H <sub>2</sub> S, HCN	1. SO <sub>2</sub> , NH <sub>3</sub> , HCN, H <sub>2</sub> S	1. Cl <sub>2</sub> 2. SO <sub>2</sub> , HCN 3. NH <sub>3</sub> , H <sub>2</sub> S 4. H <sub>2</sub> S	No difference	1. HCN 2. H <sub>2</sub> S 3. Cl <sub>2</sub> 4. SO <sub>2</sub> 5. NH <sub>3</sub>
B. Order of Sensitivity of Classes of Organisms to Gases				
Ammonia	Chlorine	Hydrogen Cyanide	Hydrogen Sulphide	Sulphur Dioxide
1. Leaves 2. Stems, Fungi and Bacteria 3. Seeds and Sclerotia* Animals	1. Leaves 2. Fungi and Bacteria, Stems, Animals 3. Seeds and Sclerotia	1. Animals 2. Leaves, Stems 3. Fungi and Bacteria, Seeds and Sclerotia	1. Animals 2. Leaves, Stems 3. Fungi and Bacteria, Seeds and Sclerotia	1. Leaves, Fungi and Bacteria 2. Stems, Animals, Seeds and Sclerotia

\* No significant difference between Seeds and Sclerotia and group 2.



the toxicity curves, comparisons made at other times and concentrations may not give necessarily the same order of difference in toxicity or sensitivity. Also the data on green plants were available only in the form of time to kill 50 per cent of the surface area (7).

The results of comparisons of  $LT_{50}$  values at 1000 p.p.m. are shown graphically in Figure 1. Details regarding this method of plotting may be found in paper II of this series (5). An examination of Figure 1 indicates that in their response to the gases the different organisms may be placed in five more or less distinct classes, namely the fungi and bacteria, seeds and sclerotia, green leaves, green stems, and animals. These comparisons are not absolute but hold only for the physiological and environmental conditions specified in the detailed reports. Nevertheless, the comparisons of the classes of organisms rest on a broad basis of differing organisms within a class.

It thus becomes of interest to compare the relative toxicity of the different gases to a particular class of organisms, and to compare the sensitivity of different classes of organisms to a given gas. However, in order to demonstrate that real differences actually exist, that is, that one class of organisms in general is more sensitive to a particular gas than another class of organisms, it is necessary that the variation within a class be significantly less than that between classes. Such a comparison has been made on the logarithm of the  $LT_{50}$  values, employing Fisher's  $t$  test (3) for the comparison of two means. In comparisons involving the "greater or less than" values, true probabilities were calculated on ranked data.

The results of these comparisons are given in detail in Table I. It will be noted that in general  $SO_2$  and  $Cl_2$  tend to be the most toxic towards plant life and HCN and  $H_2S$  the most toxic to animals. In the responses produced on similar organisms  $SO_2$  and  $Cl_2$  appear to be alike on the one hand, and HCN and  $H_2S$  on the other. The resting organs, seeds and sclerotia, are the most resistant to gases in general and green leaves and animals the most sensitive.

#### SUMMARY

1. A cooperative survey was made of the toxicity of ammonia, chlorine, hydrogen cyanide, hydrogen sulphide, and sulphur dioxide gases to 18 species including fungi and bacteria pathogenic to plants and animals, green leaves and stems, seeds, insects and rodents.

2. The apparatus employed permitted a continuous flow of gases at controlled concentrations. The various organisms were exposed to 1, 4, 16, 63, 250, and 1000 p.p.m. of gas for time periods of 1, 4, 15, 60, 240, and 960 minutes.

3. The order of toxicity of gases at 1000 p.p.m. was as follows: fungi and bacteria— $SO_2$ ,  $Cl_2 > NH_3 > H_2S$ , HCN; seeds and sclerotia— $SO_2 > Cl_2$ ,

$\text{NH}_3$ ,  $\text{HCN}$ ,  $\text{H}_2\text{S}$ ; green leaves— $\text{Cl}_2 > \text{SO}_2 > \text{NH}_3$ ,  $\text{HCN} > \text{H}_2\text{S}$ ; green stems—no difference; animals— $\text{HCN} > \text{H}_2\text{S} > \text{Cl}_2 > \text{SO}_2 > \text{NH}_3$ .

4. The order of sensitivity of classes of organisms was as follows: ammonia—leaves > stems, fungi and bacteria > seeds and sclerotia, animals; chlorine—leaves > fungi and bacteria, stems, animals > seeds and sclerotia; hydrogen cyanide—animals > leaves, stems > fungi and bacteria, seeds and sclerotia; hydrogen sulphide—animals > leaves, stems > fungi and bacteria, seeds and sclerotia; sulphur dioxide—leaves, fungi and bacteria > stems, animals, seeds and sclerotia.

5. Details of the studies on the various organisms appear in subsequent papers.

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# TOXICITY OF AMMONIA, CHLORINE, HYDROGEN CYANIDE, HYDROGEN SULPHIDE, AND SULPHUR DIOXIDE GASES.

## II. FUNGI AND BACTERIA

S. E. A. MCCALLAN AND F. R. WEEDON<sup>1</sup>

No studies on the toxicity of some of the common gases to fungi and bacteria appear to have been made in which it was possible to control adequately the concentration of the gas. Hence it was considered of interest to determine the toxicity of ammonia, chlorine, hydrogen cyanide, hydrogen sulphide, and sulphur dioxide gases to fungi and bacteria under conditions which permitted a continuous flow of gas at a controlled concentration. This apparatus is described elsewhere (11), while the general plan of the experiments is discussed in the first paper of this cooperative series (4).

### METHODS

*Organisms.* Nine different plant pathogens representing the Ascomycetes and Fungi Imperfectii, and two animal pathogens—a yeast and a bacterium—were selected for investigation in this survey. The plant pathogens were: *Sclerotinia fructicola* (Wint.) Rehm., isolated from cherry; *Ceratostomella ulmi* (Schwarz) Buisman, from elm<sup>2</sup>; *Glomerella cingulata* (St.) Sp. & von S., from apple; *Macrosporium sarcinaeforme* Cav., from red clover; *Pestalotia stellata* B. & C., from *Ilex*; *Botrytis* sp. (*cinerea* type) two strains, both isolated from peony, the one spore-producing, the other predominantly sclerotia-producing<sup>3</sup>; *Rhizoctonia tuliparum* (Kleb.) Whetzel and Arthur, from tulip<sup>4</sup>; and *Sclerotium delphinii* Welch, from delphinium<sup>5</sup>. The animal pathogens were: *Monilia albicans* Robin-Zopf freshly isolated from sputum, and a standard strain of *Escherichia coli* (Migula) Cast. & Chalm<sup>6</sup>. Other bacteria were also tested in preliminary experiments as will be indicated later. Tests were made on actively growing cultures and also on sclerotia in the case of the sclerotium-producing species.

<sup>1</sup> Director, Jamestown Municipal Laboratory, Jamestown, New York and Director, Chautauqua County Laboratory, Dunkirk, New York.

<sup>2</sup> Kindly furnished by Dr. K. G. Parker, Cornell University, Ithaca, New York.

<sup>3</sup> We are indebted to Prof. H. H. Whetzel, Cornell University, for classifying the two strains of *Botrytis* isolated from peony. The No. 1 strain producing an abundance of spores in culture and no sclerotia has been used in other studies under the incorrect name of *B. paeoniae* Oud. (See Additional Errata in Contrib. Boyce Thompson Inst., Index Vols. 1-10. 1939.)

<sup>4</sup> Identification kindly confirmed by Prof. Whetzel.

<sup>5</sup> Identification kindly confirmed by Dr. D. S. Welch, Cornell University.

<sup>6</sup> Obtained from Division of Laboratories and Research, New York State Department of Health, Albany, New York.

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*Experimental procedure.* The plant pathogenes were cultured on potato dextrose agar at  $21^{\circ}\text{C}$ . and exposed to the various gases when approximately 60 hours old. At this age there is no spore formation to complicate the results on mycelial cultures. Usually four different species were grown in one Petri dish and in all cases every species was replicated four times for each time of exposure or concentration of gas. The tops were removed from the Petri dishes and the bottoms inverted and placed on specially designed wooden racks with open bottoms. The racks were then suspended in the gas chambers. By such a procedure contaminations were reduced to less than 1 per cent. Immediately after exposure, transfers were made to sterile potato dextrose slants and the presence or absence of growth recorded after 3, 7, 14, and 28 days at  $21^{\circ}\text{C}$ .

The cultures of *Monilia albicans* and *Escherichia coli* were handled in the same general manner except that the former was cultured on wort agar at room temperature for eight hours before exposure, and the latter on methylene blue agar at  $37^{\circ}\text{C}$ . for eight hours. After exposure transfers were made onto the respective agars and the presence or absence of growth noted after approximately 8 and 24 hours and 3 to 28 days. These two organisms were exposed in triplicate cultures.

Sclerotia were obtained from different aged cultures of the *Botrytis* sp., No. 2, *Rhizoctonia tuliparum*, and *Sclerotium delphinii*, placed in open Petri dishes on the wooden racks and exposed to the gas. At the end of each time interval, 10 sclerotia were removed and placed, one each on sterile potato dextrose agar slants. The presence or absence of growth was observed after 7, 14, 30, and 60 days. In this case also the contaminations were less than 1 per cent.

As stated in the first paper (4) the concentrations of gas were 1, 4, 16, 63, 250, and 1000 parts per million of air by volume, while the organisms were exposed for periods of 1, 4, 15, 60, 240, and 960 minutes. The mean temperature was  $73.3 \pm 3.3^{\circ}\text{F}$ . for all gases except HCN where it was  $79.6 \pm 3.5^{\circ}\text{F}$ .

A given experiment performed on one day with a set of cultures produced at the same time, usually consisted of maintaining one concentration of gas and exposing the cultures for the different lengths of time. Thus another experiment at a different concentration on a different day would involve a different set of cultures and hence introduce the important day-to-day variable (6). In some cases actual repetitions of a given concentration were performed. The tests with the sclerotia were run only at 1000 p.p.m. and exposures of 1, 4, and 16 hours, i.e., 60, 240, and 960 minutes.

## RESULTS

The final results after waiting the maximum time of observation were plotted on a logarithmic scale of concentration and time for each gas and each species as illustrated in Figure 1. The response, i.e., 0, 1, 2, 3, or all 4,



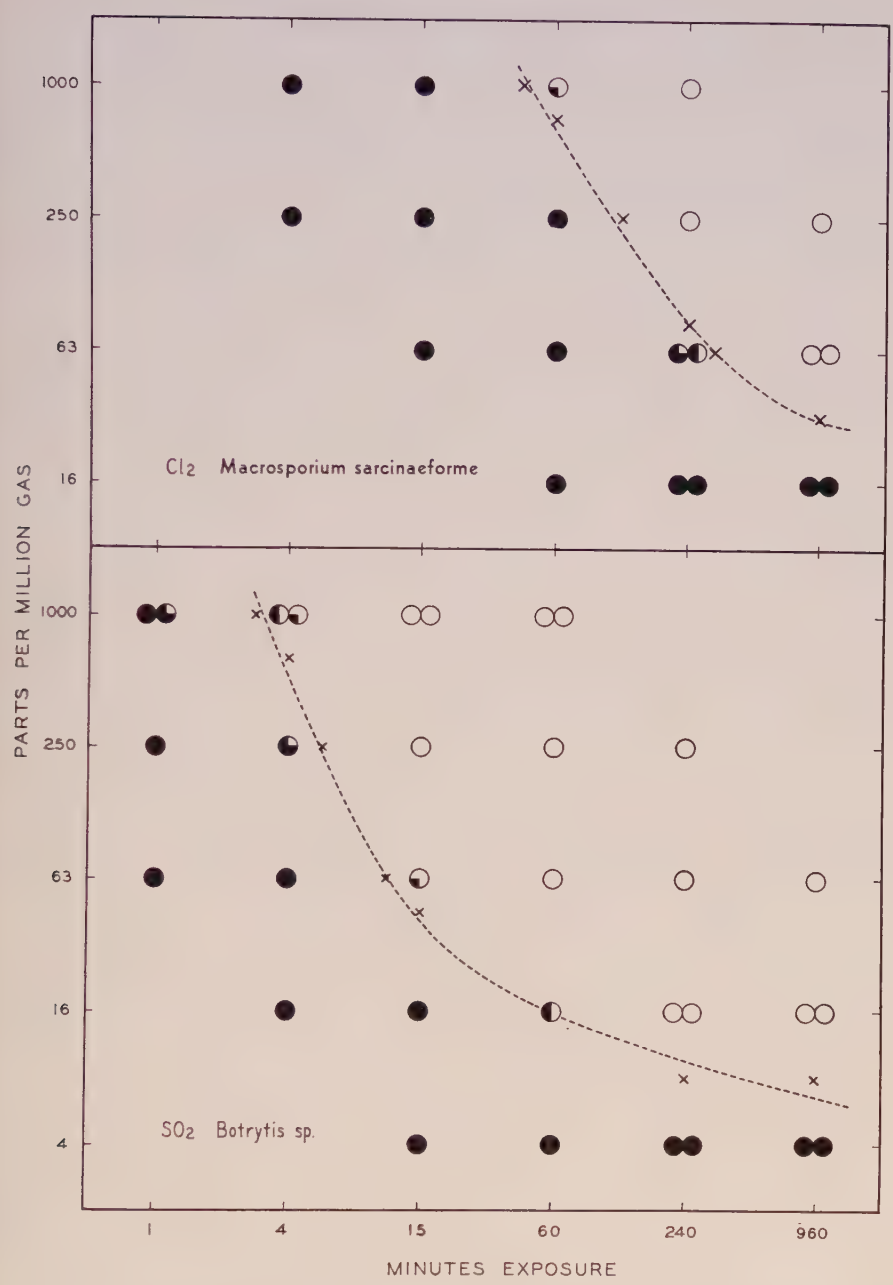


FIGURE 1. Examples of response of cultures to different concentrations and time of exposure to gases. Solid circle indicates all four cultures living, open circle all dead, and each solid quarter a living culture. Crosses indicate estimated 50 per cent kill points and dotted line the time-concentration curve for 50 per cent killed.

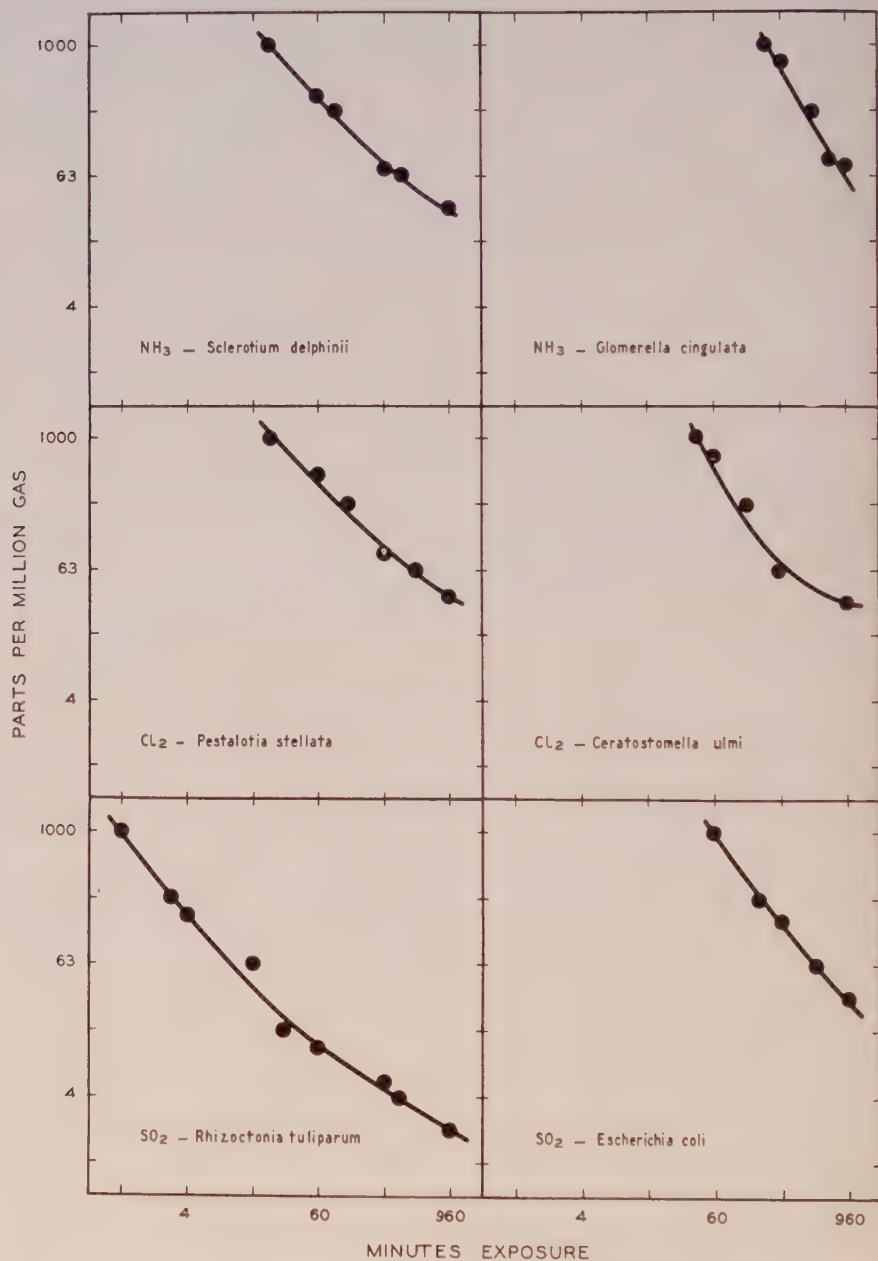


FIGURE 2. Examples of time-concentration curves for 50 per cent kill. Solid circles indicate estimated 50 per cent kill points, i.e., LD<sub>50</sub> or LT<sub>50</sub> values.

of cultures killed was indicated at each point of intersection of time and concentration, assuming a straight line relation between points, and the position for 50 per cent killed estimated graphically to the nearest quarter dose unit. By this means time-concentration curves for 50 per cent kill were obtained for each species and gas. Some typical ones are illustrated in Figure 2. It will be seen that for a given response, i.e., 50 per cent kill, there is a reciprocal relation or hyperbolic function between time and concentration. Thus the same effect may be attained at low concentrations and long times, or high concentrations and short times.

The results with the sclerotia are shown in Table I.

TABLE I

NUMBER OF SCLEROTIA GERMINATING OUT OF 10 EXPOSED TO 1000 P.P.M. OF GAS FOR 1, 4, AND 16 HRS. THREE AGES OF SCLEROTIA

Species	Age in weeks	HCN			H <sub>2</sub> S			NH <sub>3</sub>			Cl <sub>2</sub>			SO <sub>2</sub>		
		1	4	16	1	4	16	1	4	16	1	4	16	1	4	16
<i>Botrytis</i> sp. ( <i>cinerea</i> type)	2-3	10	10	10	10	10	10	—	10	10	10	10	0	—	0	0
	4-6	10	10	10	10	10	10	10	10	10	10	10	4	5	4	0
	10	10	10	10	10	10	10	10	10	10	10	9	4	10	4	0
<i>Rhizoctonia</i> <i>tuliparum</i>	2-3	10	6	6	10	10	7	10	10	3	10	3	5	4	—	—
	4-6	10	10	2	9	10	10	10	9	7	10	10	8	10	9	10
	10	10	10	5	10	10	10	10	10	10	10	10	9	10	10	10
<i>Sclerotium</i> <i>delphinii</i>	2-3	10	10	10	10	10	10	5	0	0	10	10	7	0	—	—
	4-6	10	10	10	10	10	10	4	3	2	10	9	10	4	4	2
	10	10	10	—	10	10	10	6	2	5	10	10	9	8	6	3

#### LT<sub>50</sub> VALUES AT 1000 P.P.M.

In order to compare the response of the different species to the various gases the times to attain the point of 50 per cent kill or LT<sub>50</sub> (4) for a concentration of 1000 p.p.m. have been plotted on a composite graph which is shown in Figure 3. It will be observed that the plant and animal pathogene cultures responded similarly, with SO<sub>2</sub> or Cl<sub>2</sub> being the most toxic gas, with ammonia intermediate, and hydrogen sulphide and hydrogen cyanide being relatively non-toxic. *Sclerotium delphinii* and *Rhizoctonia tuliparum* may be seen to be the most sensitive species and *Ceratostomella ulmi* and *Escherichia coli* the most resistant. The sclerotia are much more resistant to the action of the gases, and at the time and concentrations employed little difference can be shown among the gases. However, for two species, SO<sub>2</sub> appears the most toxic gas.

#### EFFECT OF AGE

Since *Rhizoctonia tuliparum* and *Sclerotium delphinii* are the slowest species to start active growth in fresh cultures, a test was run using older cultures. Instead of the usual two and one-half-day-old cultures, five- and

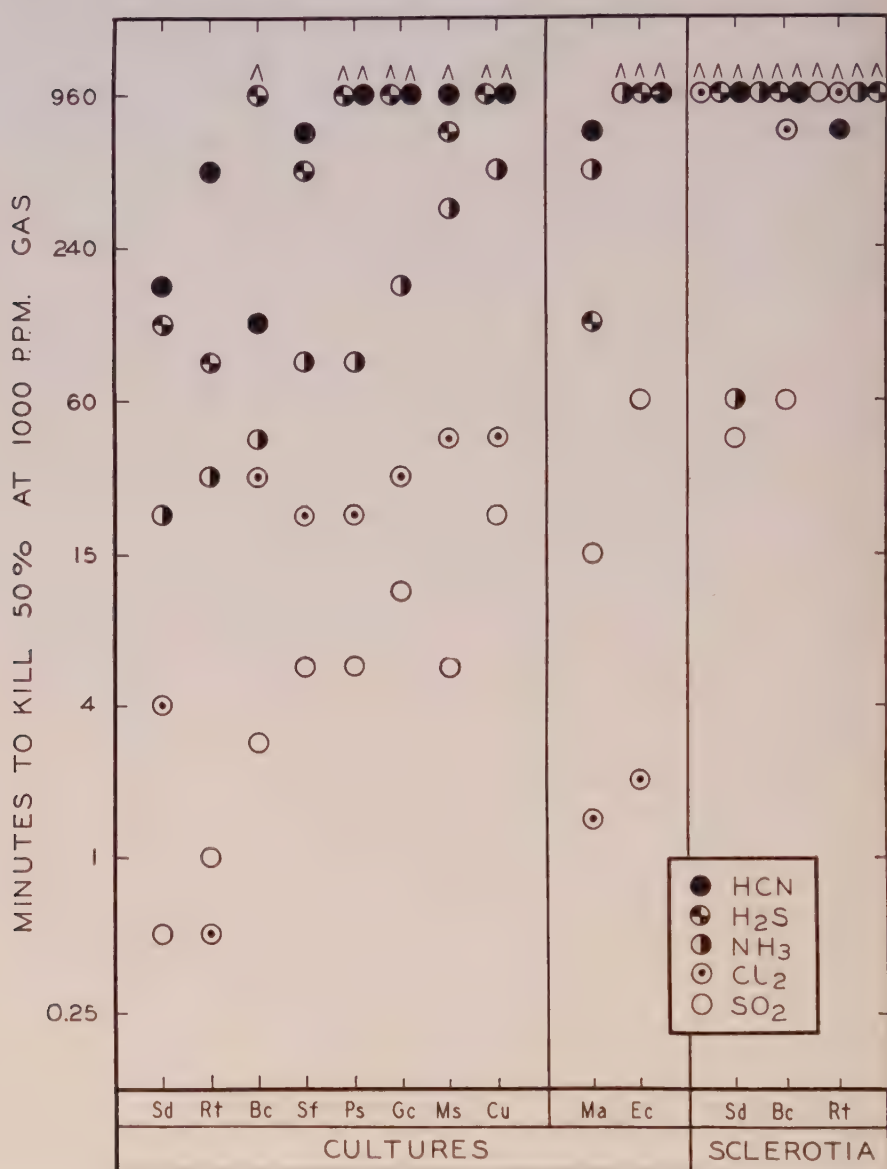


FIGURE 3. Comparison of LT<sub>50</sub> values in minutes at 1000 p.p.m. for different gases and organisms. Abbreviations of organism names from left to right as follows: *Sclerotium delphinii*, *Rhizoctonia tuliparum*, *Botrytis* sp. (*cinerea* type), *Sclerotinia fructicola*, *Pestalotia stellata*, *Glomerella cingulata*, *Macrosporium sarcinaeforme*, *Ceratostomella ulmi*, *Monilia albicans*, and *Escherichia coli*.



four-day-old cultures, respectively, were exposed to chlorine gas. Under these conditions the LT<sub>50</sub> value at 1000 p.p.m. was increased from 0.5 and 2 respectively, to 6 minutes and 4 minutes. In the case of the sclerotia, as shown in Table I, the 2 to 3 weeks old were the most sensitive and the 10 weeks the least sensitive.

#### DELAYED GROWTH

A decided delay in visible growth was observed after transferring the exposed cultures and sclerotia to fresh slants. This delay was not associated with any particular gas, concentration, or time. It was especially marked in the case of the sclerotia. Composite results are shown in Table II.

TABLE II  
DELAY IN VISIBLE GROWTH OF VIABLE CULTURES AND SCLEROTIA AFTER  
EXPOSURE TO GASES

A—Cultures, all 8 species. 280–550 cultures per gas								
			Con- trol	HCN	H <sub>2</sub> S	NH <sub>3</sub>	Cl <sub>2</sub>	SO <sub>2</sub>
	Percentage growing after	3 days	100	96.2	95.6	90.8	97.1	89.0
Additional	"	" 7 "	0	3.6	4.2	9.2	2.3	10.8
"	"	" 14 "	0	0.2	0.2	0	0.6	0.2
"	"	" 28 "	0	0	0	0	0	0

B—Sclerotia, all 3 species. 110–200 sclerotia per gas								
	Percentage growing after	7 days	100	90.0	78.1	80.5	82.6	68.0
Additional	"	" 14 "	0	9.3	18.9	18.2	12.6	27.3
"	"	" 30 "	0	0.7	3.0	1.2	4.8	3.7
"	"	" 60 "	0	0	0	0	0	0

#### pH OF MEDIA

Determinations of the pH value of the three media when exposed to 1000 p.p.m. of the different gases for the different lengths of time were made. Duplicate readings were taken with a glass electrode (13), and generally agreed closely. The average values are plotted in Figure 4. The media exposed to H<sub>2</sub>S and HCN changed but little, but that exposed to SO<sub>2</sub> and Cl<sub>2</sub> became very acid and that to NH<sub>3</sub> very alkaline.

It was thought that perhaps the apparent toxicity of SO<sub>2</sub>, Cl<sub>2</sub>, and NH<sub>3</sub> might be due to an indirect effect on the culture media. Accordingly, sterile plates of potato dextrose agar were exposed to 250 p.p.m. of the gases for 16 hours. The plates of agar were then left open in the laboratory for 12 hours to aerate and afterwards inoculated. The results of the subsequent growth are compared in Table III to that of cultures actually exposed to the same amount of gas in the usual manner. The sterile media were not rendered toxic by exposure to HCN, H<sub>2</sub>S, or NH<sub>3</sub>. In the case of

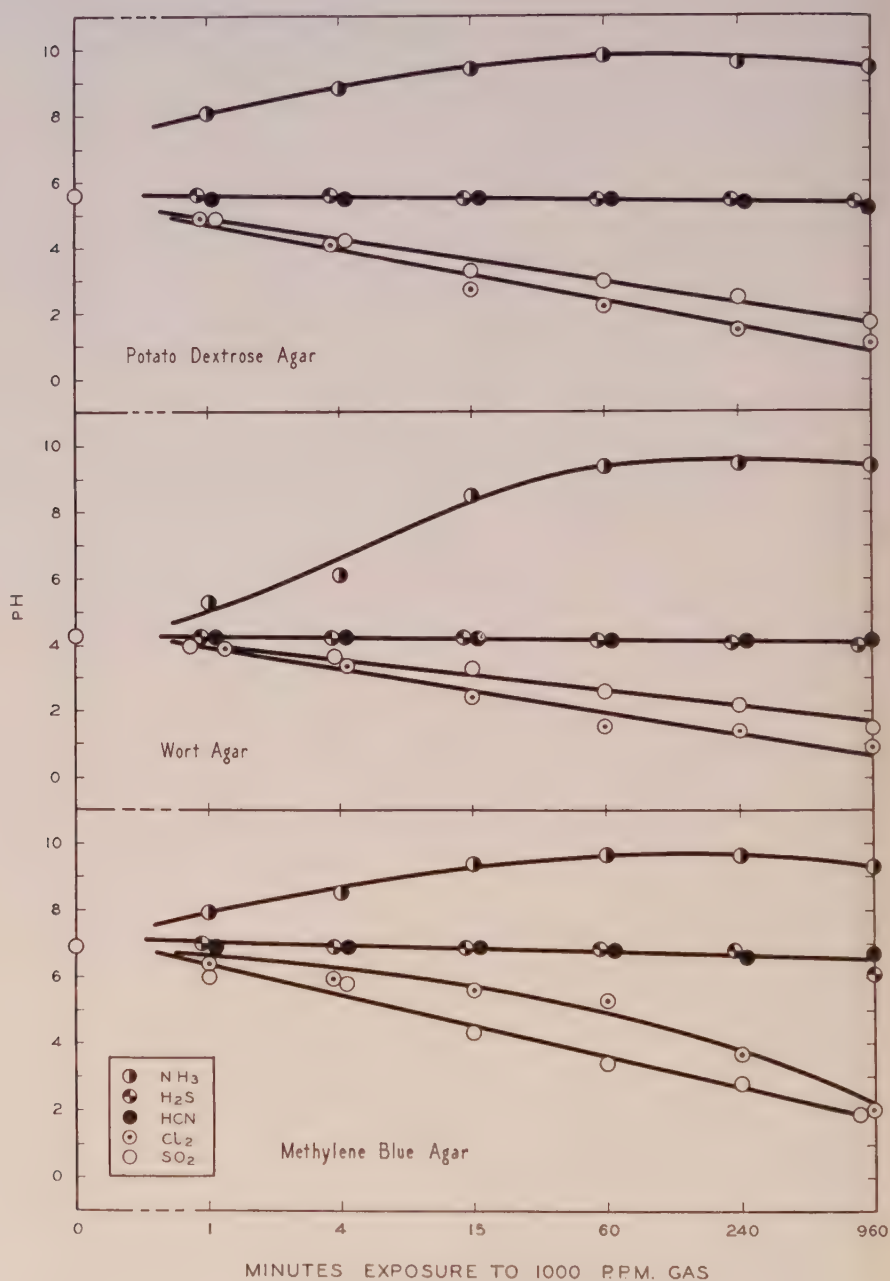


FIGURE 4. Changes in pH of sterile media when exposed to 1000 p.p.m. of different gases for different lengths of time.

several fungi the plates permitted growth after exposure to  $\text{Cl}_2$  and  $\text{SO}_2$ , whereas when cultures were exposed under these conditions they were all killed. However, the remaining fungi would not grow on the plates exposed to  $\text{Cl}_2$  and  $\text{SO}_2$ . It is concluded that under certain conditions the toxicity of the gas may be partially due to its indirect effect on the media, but in general its action must be a direct effect.

TABLE III

TOXIC EFFECT OF GAS TREATMENTS ON POTATO DEXTROSE AGAR. COMPARISON OF GROWTH (a) ON STERILE MEDIA PREVIOUSLY EXPOSED TO GAS AND SUBSEQUENTLY AERATED AND INOCULATED, WITH (b) CULTURES ON MEDIA AT TIME OF TREATMENT AND SUBSEQUENTLY TRANSFERRED TO FRESH MEDIA. GAS TREATMENTS, 250 P.P.M. FOR 16 HOURS

	HCN		$\text{H}_2\text{S}$		$\text{NH}_3$		$\text{Cl}_2$		$\text{SO}_2$	
	a	b	a	b	a	b	a	b	a	b
<i>Sclerotium delphinii</i>	++	o	++	+	++	o	o	o	o	o
<i>Rhizoctonia tuliparum</i>	++	o	++	++	++	o	o	o	o	o
<i>Botrytis</i> sp. (cinerea type)	++	+	++	++	++	o	++	o	++	o
<i>Sclerotinia fructicola</i>	++	++	++	++	++	o	++	o	o	o
<i>Glomerella cingulata</i>	++	++	++	++	++	++	o	o	o	o
<i>Macrosporium sarcinaeforme</i>	++	++	++	++	++	o	o	o	o	o
<i>Pestalotia stellata</i>	++	++	++	++	++	o	++	o	++	o
<i>Ceratostomella ulmi</i>	++	++	++	++	++	++	o	o	o	o

++ = Growth of all cultures; + = growth of some cultures; o = no growth.

#### PRELIMINARY RESULTS WITH OTHER BACTERIA

Preliminary studies exposing several species of bacteria and a variety of culture media to sulphur dioxide at different concentrations were made<sup>7</sup>. It was found that blood agar was turned chocolate brown by this gas and that the several bacteria used failed to grow upon this changed medium while growing well upon other media at the same concentration and period of exposure to the gas.

Recently isolated strains of *Streptococcus pyogenes* (Rosenbach), *Diplococcus pneumoniae* (Weichselbaum) Group IV, type XIV, and *Staphylococcus pyogenes aureus* (Rosenbach) were found to be similar to each other and to *E. coli* in resistance but extended studies with the three former were not pursued at this time due to certain difficulties in handling. A spore-forming bacillus partly identified as *Bacillus subtilis* Cohn emend Prazmowski was not killed by sulphur dioxide at the concentrations and periods studied.

#### DISCUSSION OF LITERATURE

All tests with these gases reported in the literature indicate that the gas was introduced, or chemically evolved in a closed chamber. Under

<sup>7</sup> Thanks are due to the staff of the Yonkers Bureau of Laboratories, particularly Mrs. L. A. Day and Miss F. E. D. Knacke for help in handling these cultures

these conditions it would be impossible for the concentration of the gas to remain constant since it would be changed by adsorption on the walls of the chamber, absorption in the media, or by decomposition or other chemical change. Hence such results may not be comparable with a continuous flow of gas at a controlled concentration.

The most extensive results reported are those of Tomkins (14) who observed the germination and growth of *Trichoderma lignarum* in continuous exposure. Germination and growth occurred in concentrations not greater than 2800 p.p.m. for HCN, 900 for SO<sub>2</sub>, 180 for H<sub>2</sub>S, and 100 for NH<sub>3</sub>.

Ammonia has been found by Neal and co-workers (8, 9, 10), to have a specific toxic effect on the cotton root-rot fungus. Mycelium and sclerotia exposed to presumably pure gas were killed in a few seconds. However, the sclerotia do not appear to have been examined for germination beyond three days on agar.

Leukel and Nelson (3) inquired into the possibilities of chlorine gas as a seed disinfectant, but did not find it particularly promising. Concentrations between 3 and 9 per cent for 1 to 2 hours were the most satisfactory.

The effect of hydrogen cyanide on a number of different fungi has been studied by Sibia (12). In general, growth was resumed after exposure to concentrations of several p.p.m., for 60 to 95 minutes.

Marsh (7) and McCallan and Wilcoxon (5) have demonstrated the relatively high toxicity of hydrogen sulphide to fungous spores. Following these results several workers in Russia (1, 2, 15) have reported promising laboratory indications on the effectiveness of H<sub>2</sub>S as a fumigant to control various bacterial and fungous seed-borne diseases. The time of exposure was from 48 to 168 hours at concentrations ranging from 300 p.p.m. to 50 per cent.

Since hydrogen sulphide is less soluble in water than the other gases, the original data of McCallan and Wilcoxon (expressed in mg. per liter soln.) were reexamined and the LD<sub>50</sub> values determined for the concentration in the air above the spore suspension drops. Five similar species employed in the present paper were found to have LD<sub>50</sub> values in p.p.m. at 22 hours for spore germination inhibition as follows: *Sclerotinia fructicola* 80, *Macrosporium sarcinaeforme* 150, *Pestalotia stellata* 180, *Glomerella cingulata* 1500, and *Botrytis* sp. (*cinerea* type) 3000. Similar values, in the present study, for mycelial culture inhibition at 10 hours give respectively 500, 800, >1,000, >1,000, and >1,000. The order of sensitivity is seen to be approximately the same and under the two different conditions spores are about 5 to 6 times as sensitive as mycelial cultures.

#### SUMMARY

1. Young actively growing cultures, on appropriate media, of 8 plant pathogenes, namely *Sclerotinia fructicola*, *Ceratostomella ulmi*, *Glomerella*



*cingulata*, *Macrosporium sarcinaeforme*, *Pestalotia stellata*, *Botrytis* sp. (*cinerea* type), *Rhizoctonia tuliparum*, and *Sclerotium delphinii*, and of 2 animal pathogenes, *Monilia albicans* and *Escherichia coli*, were exposed to a continuous flow of  $\text{SO}_2$ ,  $\text{Cl}_2$ ,  $\text{NH}_3$ ,  $\text{H}_2\text{S}$ , and  $\text{HCN}$  gases under controlled conditions. Exposures were made for 1, 4, 15, 60, 240, and 960 minutes at concentrations of 1, 4, 16, 63, 250, and 1000 p.p.m. After exposure the presence or absence of growth on fresh sterile media was noted.

2. Sclerotia of *Botrytis* sp. (*cinerea* type), *Rhizoctonia tuliparum*, and *Sclerotium delphinii* were likewise tested.

3. A reciprocal relation between time and concentration for a given response was noted.

4.  $\text{SO}_2$  or  $\text{Cl}_2$  was the most toxic gas toward the cultures,  $\text{NH}_3$  was intermediate, and  $\text{H}_2\text{S}$  and  $\text{HCN}$  the least toxic. There was no significant difference in the response of the animal as compared to the plant pathogenes. *Sclerotium delphinii* and *Rhizoctonia tuliparum* were the most sensitive cultures and *Ceratostomella ulmi* and *Escherichia coli* the least sensitive. The sclerotia were more resistant to the gases than the cultures.

5. Young cultures and sclerotia are more sensitive than older ones. With all plant pathogenes, and all gases, there was a conspicuous delay in the visible growth of viable cultures after transferring to fresh media. In the case of the sclerotia there was an increase in the per cent viable after 30 days.

6. Exposing the culture media to  $\text{SO}_2$  and  $\text{Cl}_2$  resulted in a decided increase in acidity, while in the case of  $\text{NH}_3$  the media became much more alkaline. This indirect effect may account for the partial toxicity of the gases, but in general it is believed that the effect is a direct one.

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# TOXICITY OF AMMONIA, CHLORINE, HYDROGEN CYANIDE, HYDROGEN SULPHIDE, AND SULPHUR DIOXIDE GASES. III. GREEN PLANTS

NORWOOD C. THORNTON AND CARL SETTERSTROM

This report is a portion of a survey of the toxicity of  $\text{NH}_3$ ,  $\text{Cl}_2$ ,  $\text{HCN}$ ,  $\text{H}_2\text{S}$ , and  $\text{SO}_2$  to living tissue. The general plan of the experimental procedure is presented in the first paper of this cooperative series (3). Although many toxicity studies have been made with most of these gases on plants or on animals this is the first occasion that such an investigation has been conducted where the gas effects on many kinds of living tissue may be correlated. In any one experiment the plants, and all other test material, were exposed to a moving air stream containing a known and constant amount of the gas being studied so that the data presented represent the effect of any one concentration of gas for each particular period of treatment. This is a marked departure from the usual type of toxicity experimentation where the test material is placed in a closed container with one dose of the gas, the concentration of which varies with time. Although it is generally known what effect some of these gases would have upon the pH of living tissue, the total effectiveness of various concentrations of each gas has not before been accurately determined because of the limitations of the methods used. Finally, the various factors having to do with the toxicity of the gases to plant tissue may be considered either separately or together, depending upon the reader's interest.

## MATERIAL AND METHODS

For these investigations tomato (*Lycopersicon esculentum* Mill.) plants 6 to 8 weeks and approximately 14 weeks old, buckwheat (*Fagopyrum esculentum* Moench.) plants 2 to 3 weeks old, and tobacco (*Nicotiana glutinosa* L.) plants 11 to 12 weeks old were selected as test material because of their availability and general use in toxicity work of this nature. All the plants were grown in four-inch pots in composted soil and in greenhouses under uniform conditions. New plantings were made at intervals in order to maintain a constant supply of plants at the stated age for each species. The plants were selected for uniformity of size and age for each experiment. Tomato plants were used in both the pH and injury investigations at all concentrations, while the buckwheat and tobacco plants were used only in the injury tests and at the highest concentrations.

The plants were exposed to a continuous flow of the various gases in an apparatus that has been fully described in a previous report (5). A concise description of this apparatus together with the procedure of these tests

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has been outlined in the first paper of this series (3). In general, the plants were exposed to gas concentrations of 1, 4, 16, 63, 250, and 1000 parts per million of air by volume for periods of 1, 4, 15, 60, 240, and 960 minutes. All of the gases used were obtained from commercial cylinders of compressed gases.

In order to maintain the temperature during treatment as uniform as possible ( $73.3^{\circ}\text{F.} \pm 3.3^{\circ}\text{F.}$  except with HCN which was  $79.6^{\circ}\text{F.} \pm 3.5^{\circ}\text{F.}$ ), the experiments were started at 5:30 p.m. and ended at 9:30 a.m. the following morning. The plants for the four-hour interval were placed in treatment at 4:00 a.m. and removed at 8:00 a.m. and the shorter periods of treatment were made between 7:30 and 9:00 a.m. In this way all the plants were exposed to daylight (except for part of the 4- and 16-hour periods) during the gas treatment. In tests where the plants were treated with the gases in total darkness the experiments were run between 9:30 p.m. and 12:30 a.m. All of these tests were made during the period of September to December inclusive.

At the end of each period of gas treatment the tomato plants were brought immediately into the laboratory for pH determinations on the juice of the tissue. The leaves were cut from the stem, rinsed with distilled water and then shaken vigorously to reduce the amount of water adhering to the surface. The leaves were next placed in a porcelain mortar and thoroughly macerated with a pestle, the juice pressed out by aid of the pestle and poured into a glass vial attached to a Beckman pH meter. The small permanently enclosed glass and calomel electrodes were dipped into the juice and the pH of the tissue extract was determined at once. The same procedure was used for the determination of the pH of the stem tissue. At least two plants were used at each period of exposure to each concentration. In every case, separate determinations were made upon the leaves and stems of each plant. In every experiment the pH changes in leaves and stems of treated plants were compared with control plants of the same lot which had been removed from the greenhouse at the same time. This was necessary since the pH of leaves of tomato plants varies with the amount of sunlight during the growing period just preceding analysis. The leaves of tomato plants grown during exceptionally bright sunshine had a pH as high as 6.28, while those grown during very cloudy weather had a pH as low as 5.73. The average control pH was 5.99. The standard deviation of replicate determinations was 0.06 pH.

The pH of the surface soil in the treated tomato pots was determined at the higher concentrations. One part of soil was mixed with two parts of distilled water and the pH of this soil solution was determined with the glass electrode.

Plants used to study degree and types of injury were removed to the greenhouse following each treatment and records of the extent of injury



were made at regular intervals thereafter. The results were estimated as percentage of leaf and stem area injured by the respective gas treatments. The maximum injury recorded for any particular pot was that used in evaluating comparative degrees of injury.

## EXPERIMENTAL RESULTS

### pH DETERMINATIONS

*Leaf tissue.* The effects of the various gases on the pH of the entire tomato leaf tissue are shown in Figure 1. The average of each duplicate determination was plotted on a logarithmic scale of minutes of exposure against pH and four concentrations of each gas are represented in this family of graphs.

Chlorine gas was found the most effective in causing a lowering of the pH of the tissue. The extreme pH changes found with chlorine treatment as well as those changes found with other gases were checked by titrations of the extracts with tenth normal alkali or acid using the macro-glass electrodes of the Beckman apparatus to determine the end point. In every case the degree of acidity determined in this manner was of the same order as the pH values determined directly with the glass electrode.

It is interesting to note in Figure 1 that 1000 p.p.m. of ammonia and sulphur dioxide caused the same change of 3.1 pH units in the alkaline and acid direction respectively. However, the lower concentrations of ammonia were not effective while sulphur dioxide and chlorine were still slightly effective even with 4 p.p.m. when treatment was carried on for 960 minutes.

Low concentrations, 1 and 4 p.p.m (not shown in Fig. 1), of  $\text{SO}_2$  and  $\text{Cl}_2$  caused some lowering of the pH of only the leaf tissue while the same concentrations of  $\text{NH}_3$ ,  $\text{H}_2\text{S}$ , and  $\text{HCN}$  caused no changes in pH from that of the control tissue. With 4 p.p.m. of  $\text{SO}_2$  the pH of the leaf tissue was reduced by 0.4 pH and for 1 p.p.m. the pH was reduced 0.3 pH during a treatment of 960 minutes. Where 4 p.p.m. of  $\text{Cl}_2$  were used for 15 and 960 minutes the pH was reduced by 0.3 and 1.0 respectively and where 1 p.p.m. of  $\text{Cl}_2$  was used for the entire period of treatment the leaf tissue was lowered only 0.2 pH.

Hydrogen cyanide was slightly effective in causing a drop in pH of the tissue if a high concentration was used over the maximum period of time, but in the shorter intervals of treatment or lower concentrations no significant changes were observed. Likewise, with hydrogen sulphide no significant changes in pH were observed with the treatments.

*Stem tissue.* As shown in Figure 2, ammonia was almost as effective in causing a change in the pH of the stem tissue as in the leaf tissue of the tomato plant. The other gases, however, caused little or no change in the pH of the stem tissue.

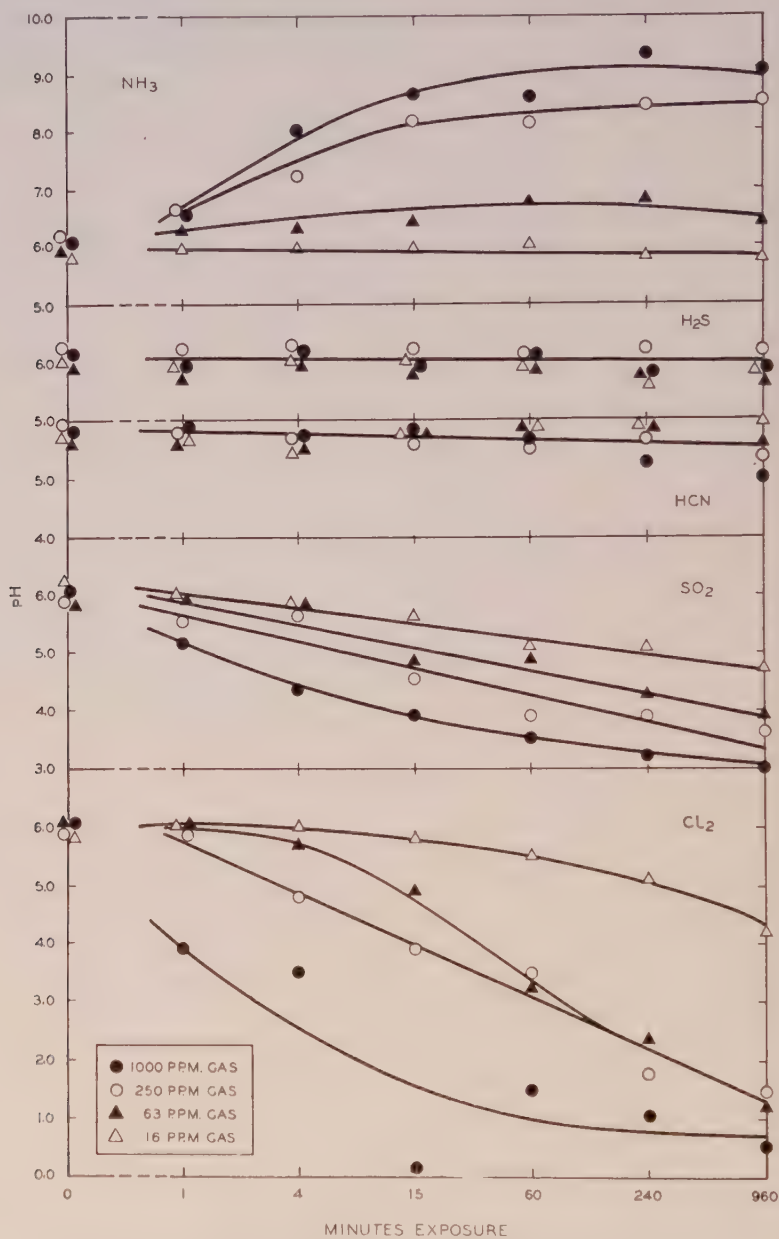


FIGURE 1. The effect of various concentrations of  $\text{NH}_3$ ,  $\text{H}_2\text{S}$ ,  $\text{HCN}$ ,  $\text{SO}_2$ , and  $\text{Cl}_2$  on the pH of leaf tissue of tomato plants exposed for different periods of time.

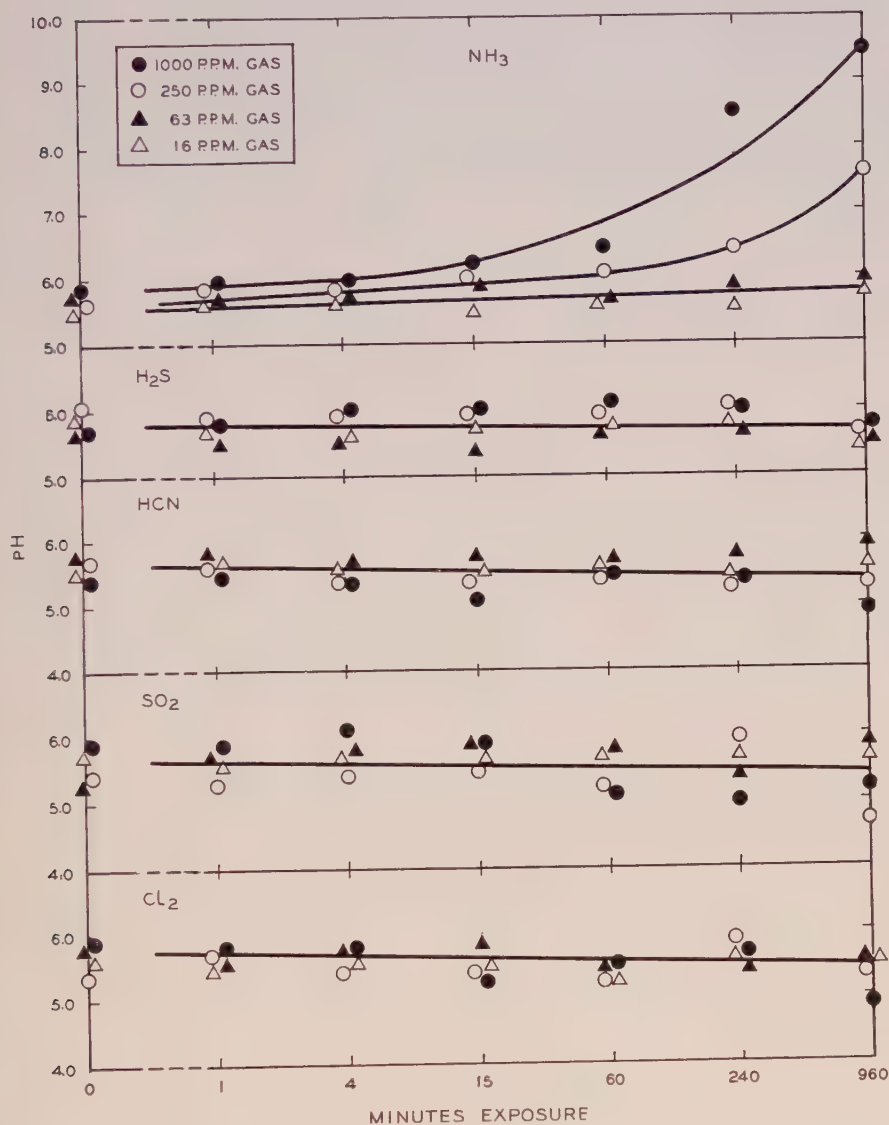


FIGURE 2. The effect of various concentrations of  $\text{NH}_3$ ,  $\text{H}_2\text{S}$ ,  $\text{HCN}$ ,  $\text{SO}_2$ , and  $\text{Cl}_2$  on the pH of stem tissue of tomato plants exposed for different periods of time.

*Special tests on leaf and stem tissue.* A few tests were made to determine the area of the plant in which the pH was affected most by the various gas treatments at a concentration of 1000 p.p.m. The area of the plant was considered in thirds, i.e., the three youngest, the three middle, and three bottom leaves, and likewise the stem was cut into three portions. In general, the preliminary tests show that with  $\text{NH}_3$  the pH of all the leaf area increases, with an indication that the older leaves may be slightly more affected than the others. In the stem tissue the youngest portion showed the greatest change. With  $\text{H}_2\text{S}$  the whole plant showed a slight drop in pH

TABLE I

RELATION OF SUNLIGHT TO THE EFFECTIVENESS OF 1000 P.P.M. OF VARIOUS GASES IN CAUSING A CHANGE IN THE pH OF TOMATO TISSUE

Gas	Minutes of treatment*	Increase (+) or decrease (−) in pH from that of control plants			
		Sunlight		Darkness	
		Leaf	Stem	Leaf	Stem
$\text{Cl}_2$	4	−2.3	−0.1	−0.1	0.0
		−3.2	−0.3	−0.5	−0.1
$\text{SO}_2$	4	−1.7	−0.2	−0.6	−0.1
		−1.6	−0.3	−0.5	−0.1
$\text{NH}_3$	240	+3.1	+3.2	+2.5	+0.9
		+3.1	+2.9	+2.5	+1.0
$\text{H}_2\text{S}$	240	−0.3	−0.3	−0.3	−0.2
		−0.5	−0.5	−0.6	0.0
$\text{HCN}$	30	−0.3	−0.1	−0.3	0.0
	60	−0.4	−0.2	−0.3	−0.1

\* Time of treatment selected to give considerable pH change with little immediate visible injury.

with the longest period of treatment. With  $\text{HCN}$  the youngest stem and leaf tissue changed the greatest with the longest period of treatment. With  $\text{SO}_2$  the results were not uniform. Middle leaves of young plants and the lowest leaves of fruiting tomato plants showed the greatest change in pH, while the stem tissue was uniform in its change in pH. In the case with  $\text{Cl}_2$ , the older or lowest three leaves always showed the greatest change in pH while the stem tissue changed uniformly from top to bottom portion.

Treatment of tomato plants in sunlight greatly increased the effectiveness of  $\text{Cl}_2$  and  $\text{SO}_2$  in causing a lowering of the pH of the tissue. The extent of these differences in pH for the gas treatments of plants held in sunlight and darkness is shown by the data in Table I. Ammonia was found to cause increase in pH of the leaf tissue irrespective of whether the plants were held in sunlight or darkness, although the greatest change was found in those



plants held in the light. This was especially noticeable in the pH of the stem tissue. Apparently light played no part in the effectiveness of  $\text{H}_2\text{S}$  and  $\text{HCN}$  in causing a slight change in pH of either the leaf or stem tissue.

Once the pH of the tomato leaf and stem tissue was changed by treatment with 1000 p.p.m. of either  $\text{Cl}_2$  or  $\text{SO}_2$  for 15 minutes or  $\text{H}_2\text{S}$  or  $\text{HCN}$  for 960 minutes there was no recovery. This was determined by treating a number of plants for the stated intervals, analyzing one pair, then removing the remaining pair to the greenhouse for various periods of time before making further determinations of the pH. Even after 26 hours the pH of the tissue remained at approximately the same value as that determined from the samples of plants when first removed from the gas treatment. However, the plants treated 60 minutes with 1000 p.p.m.  $\text{NH}_3$  responded quite differently. There was a progressive lowering of the pH (some recovery of the plant) until at the end of 24 hours in the greenhouse the pH of the tissue was 1.4 pH units below that determined on the plants at the time of removal from the gas treatment.

*Soil.* Three of the gases,  $\text{NH}_3$ ,  $\text{Cl}_2$ , and  $\text{SO}_2$ , were effective in altering the pH of the soil in which the tomato plants were growing while  $\text{H}_2\text{S}$  and  $\text{HCN}$  had no effect. In general,  $\text{Cl}_2$  concentrations of 4 to 1000 p.p.m. caused a reduction of from 0.7 to 1.6 pH below that of the soil in the control pots,  $\text{SO}_2$  concentrations of 16 to 1000 p.p.m. lowered the pH from 0.6 to 1.6, and  $\text{NH}_3$  1000 p.p.m. increased the pH 3.8 units above that of the soil from the control pots. These gases were effective only in these concentrations and during a treatment period extending from 60 to 960 minutes.

#### VISIBLE INJURY

Since the concentration of each gas was increased in a series with the ratio of 4 between successive members, and since the time intervals for the duration of the gas exposures also varied in the ratio of 4, it was particularly difficult to establish the exact time necessary for a given concentration of gas to cause injury to any species or part of a plant. The injury has been estimated as accurately as possible and the data are presented in graphs in Figures 3 and 4 for leaf and stem injury respectively. As discussed in the first paper of this series (3), the value for  $\text{LT}_{50}$  (the time necessary for any given concentration of gas to injure an estimated 50 per cent of the surface area of the plant) was chosen as a standard of comparison in this general survey of the toxic effect of these gases.

*Leaf tissue.* As shown by the family of graphs in Figure 3, the toxicity of the gases to tomato leaves may be rated in order of their decreasing effectiveness— $\text{Cl}_2$ ,  $\text{SO}_2$ ,  $\text{NH}_3$ ,  $\text{HCN}$ , and  $\text{H}_2\text{S}$ . In arranging the data it was found necessary to take into account weather conditions at the time that the plants were treated with the gases. As shown in Figure 3, the white circles and broken lines represent clear weather and the black circles and

solid lines represent cloudy weather at the time of treatment. In the case of  $\text{Cl}_2$  and  $\text{SO}_2$  it is clear that the leaf tissue was injured by any given concentration of gas in a shorter period of time on a clear day than on a cloudy day. It is interesting that this observation correlates with the effect

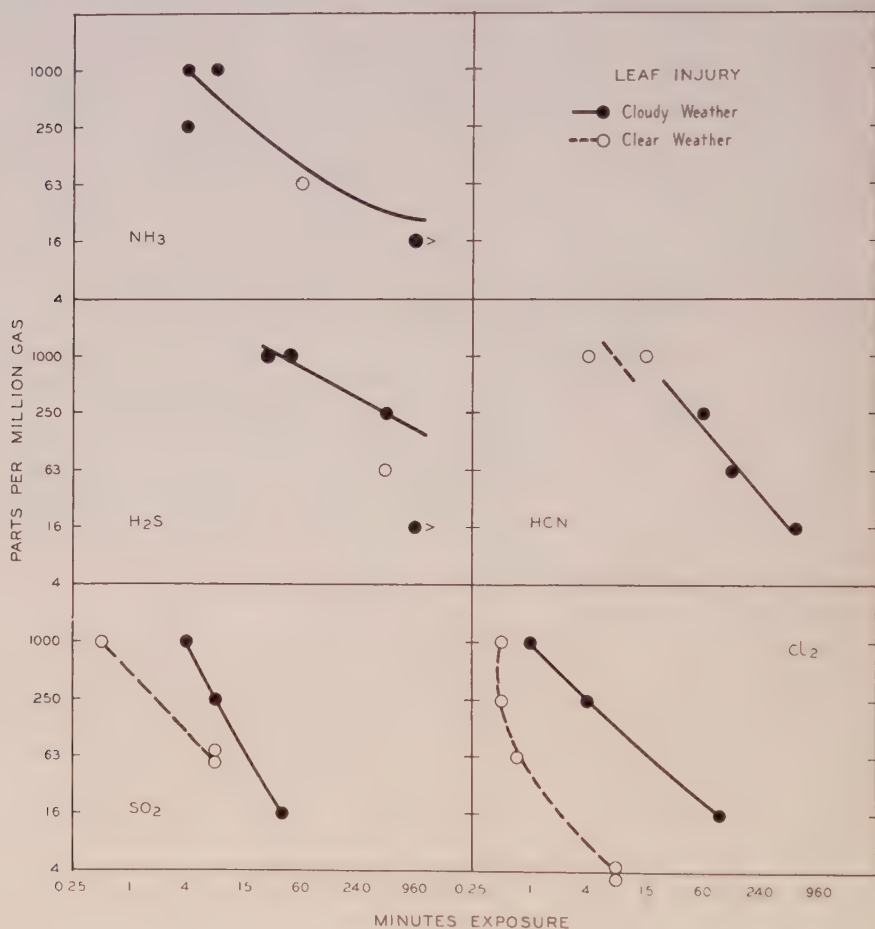


FIGURE 3. Time-concentration curves for 50 per cent of the leaf area of tomato plants to be injured by the various gases during clear and cloudy weather.

of the gas on the pH of plant tissue treated in sunlight or in darkness. Insufficient observations preclude any such statement in the case of the other gases, although the available data are represented in these graphs.

*Stem tissue.* The stems of the tomato plants were injured by  $\text{HCN}$ ,  $\text{SO}_2$ ,  $\text{Cl}_2$ ,  $\text{H}_2\text{S}$ , and  $\text{NH}_3$  in decreasing order of toxicity as shown in the graphs in

Figure 4. Here again treatment with  $\text{SO}_2$  in light was effective in shortening the time of exposure necessary to cause injury to the stems.

*Other plants.* Buckwheat and tobacco plants were tested for injury by the various gases at the same time that the tomato plants were treated.

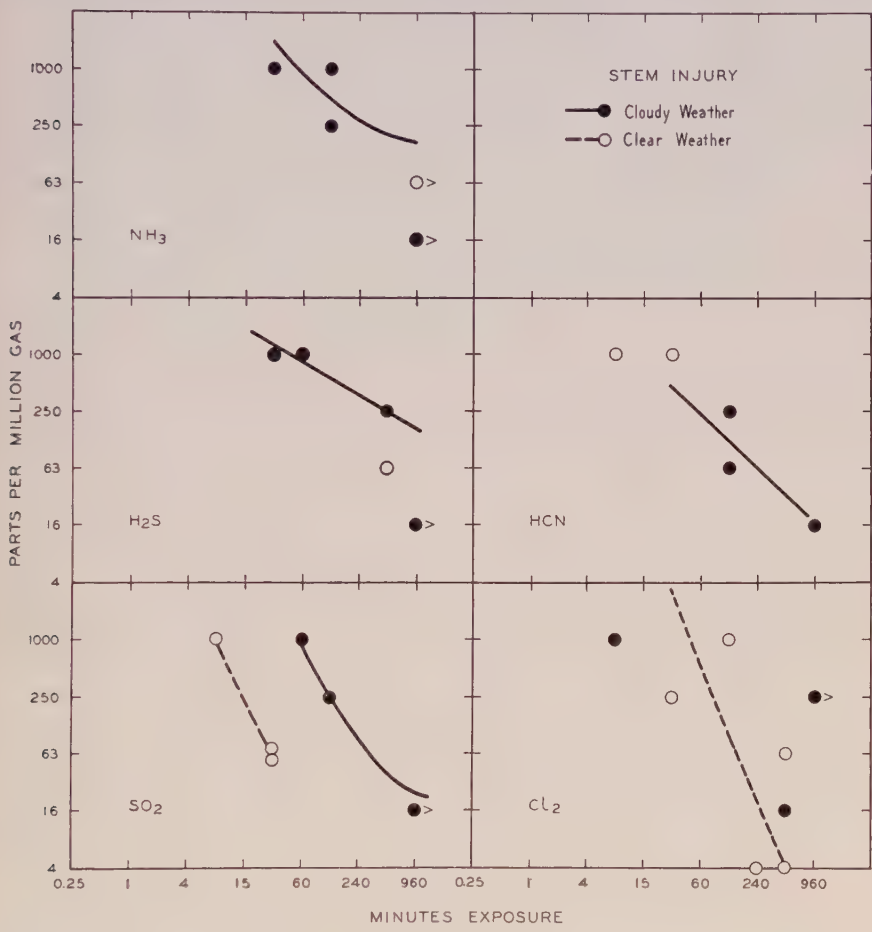


FIGURE 4. Time-concentration curves for 50 per cent of the stem area of tomato plants to be injured by the various gases during clear and cloudy weather.

The data in Table II show the time necessary to expose the plants to 1000 p.p.m. of the various gases in order to injure 50 per cent of the exposed surface of the leaves or the stems. In general, the gases were about equally toxic to each species of plant except in the case of the tobacco leaves or stems which were much more susceptible to  $\text{HCN}$  injury than either tomato or buckwheat.

TABLE II

TIME IN MINUTES TILL 50 PER CENT INJURY TO EXPOSED PLANT SURFACES AT 1000 P.P.M.

	Plant	Gas				
		NH <sub>3</sub>	Cl <sub>2</sub>	HCN	H <sub>2</sub> S	SO <sub>2</sub>
Leaves	Tomato	3	0.8	12	30	1.5
	Buckwheat	5	<4	11	60	<4
	Tobacco	8	0.5	4	100	1.5
Stems	Tomato	60	22	15	45	22
	Buckwheat	30	120	60	120	60
	Tobacco	240	60	15	480	480

## DESCRIPTION OF INJURY

*General.* Because of the 4-fold dose ratio of concentration and time it was impossible to determine accurately the progress of injury caused by the very toxic gases, Cl<sub>2</sub>, SO<sub>2</sub>, and NH<sub>3</sub>, since many of the plants were too badly injured, others not injured at all, and only relatively few were in the intermediate stages. Likewise, it was difficult to describe all the symptoms of injury resulting from each treatment. Since many investigators have reported the injurious symptoms of NH<sub>3</sub>, Cl<sub>2</sub>, H<sub>2</sub>S, and SO<sub>2</sub> on plants, little attention was devoted to this phase in the present investigation. For a detailed description of the action of these gases, reference is made here to a few of the many papers that are available.

Zimmerman and Crocker (8) have described SO<sub>2</sub> injury to many species of plants. Setterstrom and Zimmerman (6) determined the effect of environmental factors on susceptibility of plants to injury by SO<sub>2</sub>. McCallan, Hartzell, and Wilcoxon (2) described H<sub>2</sub>S injury to plants. Stout (7) has described Cl<sub>2</sub> injuries to vegetables and vegetation. Noack (4) and Brede-man and Radeloff (1) have discussed NH<sub>3</sub> injury to plant tissue. In an unpublished report on NH<sub>3</sub> and Cl<sub>2</sub> injury to plants, Dr. P. W. Zimmerman described the markings on plant leaves caused by various concentrations of these gases. Ammonia in concentrations of 1 part in 25,000 to 1 part in 120,000 caused injury to plants within a few minutes to a few hours, depending upon the concentration and period of exposure. The injury was manifested by a discoloration of the leaves which finally browned upon standing in air. Chlorine in concentrations of 0.46 to 2.95 p.p.m. for 20 to 480 minutes caused injury first to the older leaves with the development of intervenal chlorosis followed by a bleaching and entire breakdown of the leaf tissue. This progress of the development of Cl<sub>2</sub> injury was similar in many respects to the injury caused by SO<sub>2</sub>. In the present experiments where much higher concentrations of Cl<sub>2</sub> were used, the stem tissue was bleached, had a cooked appearance, and upon standing in air for a few days the damaged tissue began to dry.





FIGURE 5. Effect of HCN on tomato plants: (A) epinasty, hyponasty, and curling of leaves during exposure to 16 p.p.m. for 60 minutes; (B) injury to petioles caused by 250 p.p.m. for 60 minutes; (C) Progress of injury from petioles to stem causing water-soaked areas preceding a complete collapse of the stem during 5 days holding in greenhouse following a treatment with 1000 p.p.m. for 15 minutes.

*Hydrogen cyanide.* In these tests it was observed that HCN was particularly toxic to the stem tissue of tomato plant. At first it was found during the 15- and 60-minute treatment with 16 p.p.m. (or any comparable time-concentration treatment) that the HCN caused the tomato plants to display both epinasty and hyponasty together with curling of the leaves (Fig. 5 A). With longer or shorter periods of treatment this effect was not observed. As the period of treatment was extended or higher concentrations of HCN were used, the tip or actively growing tissue of the tomato plant became injured and petiole breakdown was progressively worse with the duration of the exposure. The HCN appeared to act specifically on the petiole and stem tissue since many of the larger leaves appeared normal (except for drying) even though the petiole was first flaccid, then collapsed, and finally appeared devoid of water and soft tissue. It was of special interest that even though the plant appeared to be only slightly injured when removed from the HCN treatment there was a continuous and decidedly progressive development of the injury. The petiole finally bent downward due to the weight of the intact leaves until it collapsed near the main stem, as shown in Figure 5 B. There next developed in the stem tissue adjacent to the petiole a water-soaked appearance (Fig. 5 C). The tissue in this area soon collapsed and the stem bent over because of the weight of the still intact but wilted leaves. This was followed by the stem drying out and the leaves dying, apparently due to the lack of water supply and some progressive development of the injury. Within a short period of time all the plants that were visibly injured by HCN at the time of removal from the treating chambers were dead because of this continued development or actual spread of the injury. This was entirely different from that observed with the plants injured by the other gases since those plants showed no tendency to increase the injured area but instead were in most cases able to recover and initiate growth if not completely injured at the time of removal from the treatments.

#### DISCUSSION

In general, there is some correlation between pH change and observed injury with  $\text{NH}_3$ ,  $\text{Cl}_2$ , and  $\text{SO}_2$ , but this is not entirely true with HCN and  $\text{H}_2\text{S}$ . Hydrogen sulphide was only mildly toxic to plant tissue as compared with the other gases. In all tests HCN was very toxic to the stems of the plants yet it caused relatively small changes in the pH of the tissue. It is apparent that the toxicity of this gas to plant tissue involves fundamental changes in the metabolism of the plant that are not readily detected by a determination of the pH of the tissue. This is especially true when we consider that HCN injury is progressive, even after removal from the gas treatment, until the entire plant is destroyed.

## SUMMARY

In order to study the toxicity of various gases to plant tissue, tomato (*Lycopersicon esculentum* Mill.), buckwheat (*Fagopyrum esculentum* Moench.), and tobacco (*Nicotiana glutinosa* L.) plants were exposed to a continuous flow of controlled concentrations of gaseous  $\text{NH}_3$ ,  $\text{Cl}_2$ ,  $\text{HCN}$ ,  $\text{H}_2\text{S}$ , and  $\text{SO}_2$ . The tomato was selected as the principal test plant and was exposed for periods of 1, 4, 15, 60, 240, and 960 minutes to concentrations of 1, 4, 16, 63, 250, and 1000 p.p.m. of each of the gases. Half the number of plants from each treatment were used for pH determinations of expressed leaf and stem juice by the glass electrode, and the other half were observed for injury development. The buckwheat and tobacco were used only to determine degree and type of injury at the higher concentrations.

The order of toxicity of the gases to the leaves and the order of effect of the gases on pH of tomato leaves was  $\text{Cl}_2 > \text{SO}_2 > \text{NH}_3 > \text{HCN} > \text{H}_2\text{S}$ .

Acidification of the leaf tissue was brought about by treatment with  $\text{Cl}_2$ ,  $\text{SO}_2$ ,  $\text{HCN}$ , and  $\text{H}_2\text{S}$ . Treatment with 1000 p.p.m. lowered the pH as follows:  $\text{Cl}_2$  from 5.8 to 4.0 in one minute and to less than 1.0 in 960 minutes;  $\text{SO}_2$  from 6.0 to 5.1 in one minute and to 2.9 in 960 minutes;  $\text{HCN}$  from 5.9 to 5.0 in 960 minutes; and  $\text{H}_2\text{S}$  from 6.1 to 5.9 in 960 minutes. The pH of the stem tissue was lowered by treatment with 1000 p.p.m. of gas for 960 minutes as follows:  $\text{Cl}_2$  0.9;  $\text{SO}_2$  0.7;  $\text{HCN}$  1.0; and  $\text{H}_2\text{S}$  0.1.

One thousand p.p.m. of  $\text{NH}_3$  caused an increase in pH of the leaf tissue as follows: 0.8 in one minute, 2.0 in four minutes, and 3.1 in 960 minutes; and of the stem tissue as follows: 0.1 in one minute, 0.2 in four minutes, and 3.6 in 960 minutes.

Titration of the plant juices with tenth normal alkali or acid allowed for the calculation of pH values of the same order as those determined by the glass electrode.

Preliminary tests were made to determine the area of the plant at which initial and maximum pH changes took place.

Tomato plants held in sunlight were found to give greater changes in pH with  $\text{Cl}_2$  and  $\text{SO}_2$  treatments than when held in darkness. Other gases— $\text{NH}_3$ ,  $\text{HCN}$ , and  $\text{H}_2\text{S}$ —were equally effective in either light or darkness.

Only with  $\text{NH}_3$ -treated plants was there any indication of a recovery toward normal in the pH of the plants upon standing in the greenhouse following treatment.

Only  $\text{NH}_3$ ,  $\text{Cl}_2$ , and  $\text{SO}_2$  were found to have an effect upon the pH of the soil in which the plants were growing.

Visible injury to plants was observed to correlate with the effectiveness of  $\text{Cl}_2$ ,  $\text{SO}_2$ ,  $\text{NH}_3$ , and  $\text{H}_2\text{S}$  in bringing about a change in the pH. With  $\text{HCN}$  the resulting injury to the plant was always greater than the pH change of the treated tissue would indicate.

The tomato plants showed both epinasty and hyponasty response to HCN which was followed by injury to the petioles. The HCN injury to the petiole tissue progressed after removal from the gas until the whole plant was destroyed.

Plants were injured by  $\text{Cl}_2$  and  $\text{SO}_2$  to a greater extent during clear than during cloudy weather.

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# TOXICITY OF AMMONIA, CHLORINE, HYDROGEN CYANIDE, HYDROGEN SULPHIDE, AND SULPHUR DIOXIDE GASES.

## IV. SEEDS

LELA V. BARTON

### INTRODUCTION

The effects on germination of seeds following exposure to certain gases applied continuously at known concentrations have been studied. The general procedure was that described by McCallan and Setterstrom (3).

For these tests radish seed, *Raphanus sativus* L., variety French Breakfast, graded as to size, and spring rye seed, *Secale cereale* L., were used. These seeds were exposed to ammonia, chlorine, hydrogen cyanide, hydrogen sulphide, and sulphur dioxide in concentrations of 250 and 1000 parts per million for periods of 1, 4, 15, 60, 240, and 960 minutes. Dry and soaked seeds were used in each test. "Soaked" seeds were placed on moist filter paper in 9 cm. Petri dishes in a constant-temperature chamber of 20° C. for four hours preceding the beginning of the exposure period. Control lots for these seeds were moistened at the beginning of the soaking period. Dry seeds were placed on moist filter paper after exposure to gases at which time control lots of untreated seeds were also placed on moist filter paper so that rates of germination might be compared.

The percentages for the dry controls as they appear in Tables I and II are averages of the four controls moistened at various times. There was very close agreement in the figures obtained for the four dry controls and the soaked control throughout the tests.

One hundred seeds each were used for radish and 50 seeds each for rye. All cultures were placed at 20° C. for germination.

### RESULTS

#### HYDROGEN CYANIDE

No reduction in the final germination percentage was evident from any of the treatments of radish seeds with this gas (Table I). However, a definite delay in germination was noted. This delay increased with increased time of exposure, was greater for the concentration of 1000 p.p.m. than for 250 p.p.m., and was especially marked for the seeds which had been soaked before exposure to the gas. Exposure for one minute to 1000 p.p.m. had no retarding effect on germination but exposures for 4, 15, 60, 240, and 960 minutes delayed the appearance of the first seedlings from pre-soaked seeds for 6, 20, 20, 30, and 67 hours respectively. These effects are shown graphically in Figure 1. The zero point is the time at which germination was first noted. Exposures to 250 p.p.m. had similar delaying effects

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in all cases except the 960-minute exposure where the delay was only 46 hours. This delay in germination occurred in spite of the fact that, for the period of exposure to HCN, the seeds were subjected to a temperature of 27° C. (3) as compared to 20° C. for the control lots. Both radish and rye seeds give high germination percentages at constant temperatures of 20°, 25°, or 30° C., but germination proceeds more rapidly at 25° or 30° C. than at 20° C., when no other factor is involved.

TABLE I

GERMINATION PERCENTAGES FROM RADISH SEEDS TREATED WITH VARIOUS GASES.  
ONE HUNDRED SEEDS WERE USED FOR EACH TEST

Treatment			Per cent germination after treatment for minutes						
Gas	Seeds	P.p.m.	0	1	4	15	60	240	960
HCN	Dry	1000	96	96	97	96	98	97	91
		250	97	93	90	92	93	99	95
	Soaked	1000	99	99	96	95	96	90	90
		250	96	97	92	97	93	90	90
H <sub>2</sub> S	Dry	1000	97	93	98	93	94	96	88
		250	96	97	96	91	96	98	96
	Soaked	1000	96	99	94	91	96	92	96
		250	96	99	95	93	96	96	95
NH <sub>3</sub>	Dry	1000	94	99	97	94	95	92	90
		250	96	94	96	100	99	93	97
	Soaked	1000	94	94	97	94	89	61	0
		250	96	93	97	95	96	94	94
Cl <sub>2</sub>	Dry	1000	96	91	92	98	98	91	94
		250	95	96	97	97	98	92	95
	Soaked	1000	95	98	93	95	97	94	5
		250	95	94	96	99	95	97	79
SO <sub>2</sub>	Dry	1000	96	92	98	97	96	93	95
		250	95	95	99	97	97	93	87
	Soaked	1000	95	94	97	95	45	0	0
		250	95	99	95	98	95	0	0

Dry radish seeds exposed to this gas for as long as 960 minutes also exhibited some delay in germination but shorter periods had no effect.

The germination of soaked rye seeds was not retarded as much as radish since 1000 p.p.m. for 1 and 4 minutes had no effect and 15, 60, 240, and 960 minutes caused delays of 4, 11, 11, and 49 hours respectively. Two hundred and fifty p.p.m. caused less retardation, no delay being evident after exposure periods of less than 240 minutes. However, exposure for as long as 960 minutes to this gas in either of the two concentrations tried

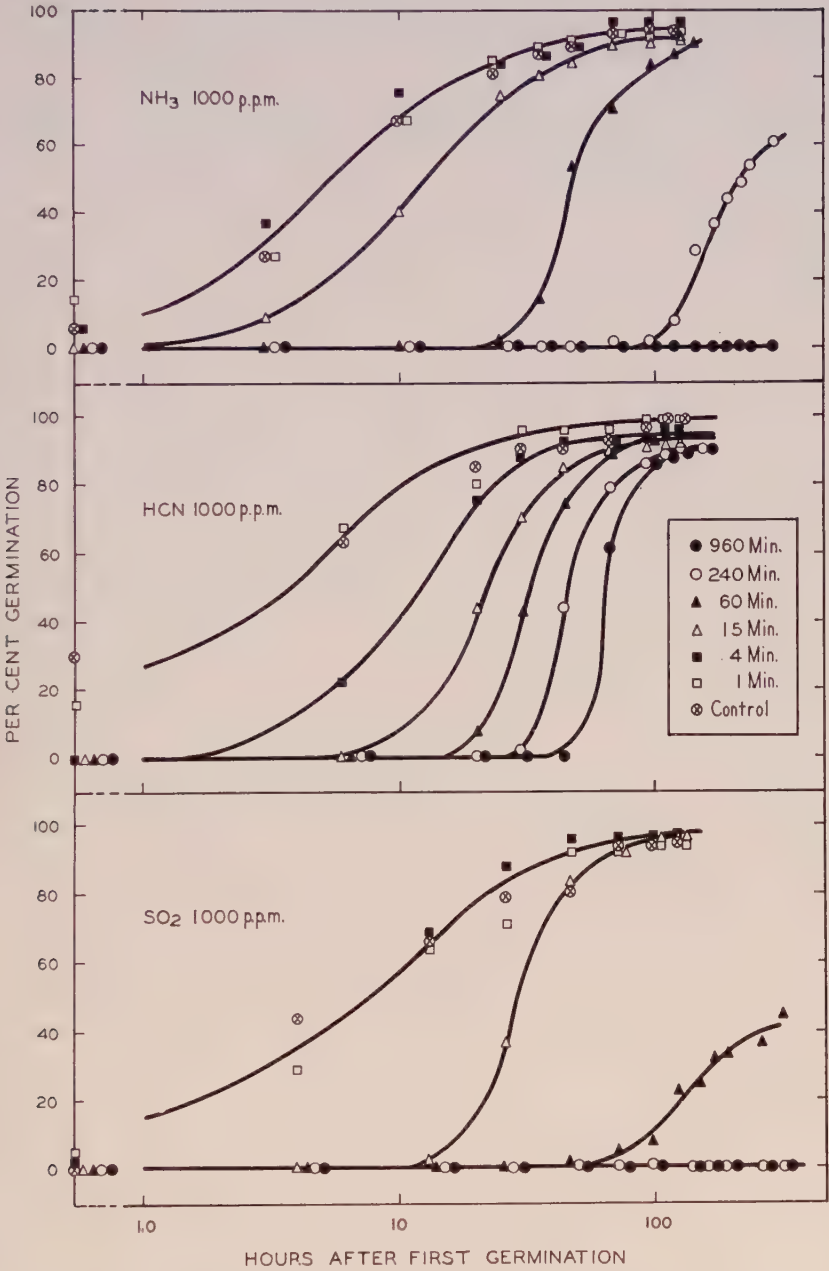


FIGURE 1. Germination rate of soaked radish seeds as affected by exposure to ammonia, hydrogen cyanide, and sulphur dioxide.

reduced the final germination obtained (Table II). This effect was not noted for radish. Dry seeds of rye were only slightly affected by hydrogen cyanide.

TABLE II

GERMINATION PERCENTAGES FROM RYE SEEDS TREATED WITH VARIOUS GASES.  
FIFTY SEEDS WERE USED FOR EACH TEST

Treatment			Per cent germination after treatment for minutes						
Gas	Seeds	P.p.m.	0	1	4	15	60	240	960
HCN	Dry	1000 250	90 92	90 86	96 80	90 98	82 86	92 88	94 86
	Soaked	1000 250	90 100	92 94	94 94	94 90	94 90	86 78	78 56
H <sub>2</sub> S	Dry	1000 250	92 87	90 88	100 96	90 90	88 86	86 96	91 88
	Soaked	1000 250	100 86	84 96	90 92	92 98	88 90	90 82	90 88
NH <sub>3</sub>	Dry	1000 250	85 87	86 90	96 84	90 88	92 86	92 94	84 90
	Soaked	1000 250	90 86	86 88	94 92	92 96	98 88	0 86	0 48
Cl <sub>2</sub>	Dry	1000 250	93 88	90 82	90 88	94 86	86 92	86 92	40 76
	Soaked	1000 250	88 82	90 88	90 84	92 78	68 80	18 56	0 14
SO <sub>2</sub>	Dry	1000 250	93 88	86 90	90 94	86 88	92 88	92 82	20 56
	Soaked	1000 250	88 82	94 86	94 88	52 80	0 6	0 0	0 0

There was some evidence of a stimulatory effect when moist seeds of either radish or rye were exposed for one minute to 250 p.p.m. of this gas. This was noticeable when the size of seedlings in the early stages of germination were compared with their controls.

Zanotti (4) also found that hydrocyanic acid gas exercised an inhibiting or stimulating action on seeds of *Datura stramonium* and *Hyoscyamus niger* according to the duration of fumigation and the mode of operation.

#### HYDROGEN SULPHIDE

Again, as in the preceding case, it was found that this gas has a relatively low toxicity as far as seeds are concerned. The final germination percentages obtained from both radish and rye seeds after all exposures compared favorably with those of the control lots (Tables I and II).



Furthermore, dry seeds of both types exposed to this gas exhibited no delay in germination. However, the germination of soaked seeds of both radish and rye was delayed 4 to 6 hours after 240 minutes' exposure to 1000 p.p.m. and 21 to 24 hours after 960 minutes' exposure to the same concentration. A concentration of 250 p.p.m. had no delaying effect on soaked radish seeds but an exposure of 960 minutes resulted in a 28-hour delay in germination of soaked rye seeds.

From the standpoint of retardation of germination, then, hydrogen sulphide was less toxic than hydrogen cyanide but neither gas prevented germination under the conditions of this experiment.

These results are in agreement with those obtained by Edelman (1) who tested the seeds of 24 varieties of field crops for their resistance to hydrogen sulphide and came to the conclusion that most of them did not respond to the gas. However, he found an increase in germinative capacity in some instances which was not shown by the present study. He used dosages of from 200 to 500 g. per cubic meter and exposures of 24 to 48 hours.

#### AMMONIA

This gas proved more injurious than either hydrogen cyanide or hydrogen sulphide. The germination of soaked seeds of radish exposed for as long as 240 minutes to 1000 p.p.m. of this gas was not only delayed but actually reduced (Table I and Fig. 1). An extension of the treatment period to 960 minutes killed all of the seeds. One minute's exposure to this same concentration, however, had no retarding effect. In fact, there seemed to be a slight stimulation of germination resulting from this treatment since the seedlings produced were slightly larger than those in corresponding controls in the hours immediately following germination. This beneficial effect was even more noticeable after the one-minute exposure to the lower concentration of 250 p.p.m. It should be kept in mind that these results are from preliminary tests only and do not include soil plantings of the treated seeds so that further tests are needed to confirm this possible stimulatory effect.

The germination of seeds treated in the dry state was delayed by 240 minutes' and 960 minutes' exposure to this gas.

Rye seeds were more sensitive than those of radish. Exposures of soaked seeds to 1000 p.p.m. for as long as 240 minutes resulted in 100 per cent kill while those exposed to 250 p.p.m. for 960 minutes had a germination capacity of only 48 per cent. Here, again, as for radish, the questionable stimulatory effect of the one-minute treatment of moist seeds was noted.

#### CHLORINE

In its toxicity to radish seeds, chlorine was similar in effects to ammonia, except that the lower gas concentration, 250 p.p.m., reduced the

germination of soaked seeds exposed for 960 minutes in this case, whereas this same treatment caused delay only, when ammonia was used.

Again, rye seeds were more sensitive than radish to this gas (Tables I and II). Bleaching resulted from the 960-minute exposure of soaked seeds to both 1000 and 250 p.p.m.

Leukel and Nelson (2) found that seeds of wheat, oats, and barley were not injured by treatment with chlorine in a concentration range of 3 to 9 per cent for 1 to 2 hours where the volume of pure chlorine gas was not more than 40 per cent of the net volume of seed being treated.

#### SULPHUR DIOXIDE

Of the five gases tried, sulphur dioxide proved most toxic to soaked seeds of radish. The germination of these seeds was reduced one-half by 60 minutes' treatment with 1000 p.p.m. and no seedlings were produced after 240 or 960 minutes' exposure to either 1000 or 250 p.p.m. (Table I and Fig. 1). Rye seeds also demonstrated the high toxicity of this gas to soaked seeds. Fifteen minutes' exposure to 1000 p.p.m. reduced the germination approximately one-half and 60 minutes' exposure killed the seeds. Two hundred and fifty p.p.m. was only slightly less toxic (Table II). A bleaching effect was noted for exposures of 60 minutes or longer for both concentrations of this gas.

#### SUMMARY

Moist and dry seeds of radish and rye were exposed to a continuous flow of HCN, H<sub>2</sub>S, NH<sub>3</sub>, Cl<sub>2</sub>, and SO<sub>2</sub> gases in concentrations of 1000 and 250 parts per million for periods of 1, 4, 15, 60, 240, and 960 minutes.

*Soaked seeds.* Moist seeds were much more sensitive to these gases than dry seeds. Delay in germination due to treatment was the principal effect noted. This delay was sometimes accompanied by reduction in germination percentage and in some instances all the seeds were killed.

Hydrogen cyanide and hydrogen sulphide had no effect on germination percentage, but delayed the appearance of seedlings under some conditions. Ammonia and chlorine were more toxic while sulphur dioxide proved most toxic under the conditions of this experiment.

A possible stimulatory effect on speed of germination was noted after exposure for one minute to 250 p.p.m. of hydrogen cyanide and either 1000 or 250 p.p.m. of ammonia. Further experiments are needed to confirm this.

A bleaching effect was evident after the 960-minute exposure of soaked seeds to 1000 or 250 p.p.m. of chlorine and after exposures of 60 minutes or longer to 1000 or 250 p.p.m. of sulphur dioxide.

*Dry seeds.* In no case did exposure of dry seeds of radish to the above gases cause reduction in germination percentage. However, such a reduction did result when dry rye seeds were exposed for 960 minutes to chlorine

or sulphur dioxide. Delay in germination was evidenced after exposure of dry seeds of both radish and rye to one or more of the longer periods in all gases except hydrogen sulphide.

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# TOXICITY OF AMMONIA, CHLORINE, HYDROGEN CYANIDE, HYDROGEN SULPHIDE, AND SULPHUR DIOXIDE GASES. V. ANIMALS

F. R. WEEDON,<sup>1</sup> ALBERT HARTZELL, AND CARL SETTERSTROM

To paraphrase Henderson and Haggard (6, p. 15), the reactions between living organisms and the gases that surround them are so immediate, so continual, and so much a matter of course that their importance is not generally appreciated. Even data on the toxicity of common industrial gases are meager and scattered, i.e., limited in regard to the gases studied, concentrations considered, methods employed, and organisms tested.

The present paper deals with one phase of a cooperative series of experiments designed to present a broader picture of gas toxicity. The plan of the experiments and a general correlation of the results on all 18 organisms included in the study are presented by McCallan and Setterstrom (9). Details are given in the other papers of the series as follows: II. Fungi and bacteria, III. Green plants, and IV. Seeds.

The toxicity of ammonia, chlorine, hydrogen cyanide, hydrogen sulphide, and sulphur dioxide to animals is perhaps of particular interest to the many important industries where these gases constitute a major health hazard. Some of these gases are also of interest as insecticides. Several noteworthy reports on the toxicity of one or the other of these gases have appeared in the literature, but most of the experimental data reported are based on exposures in air-tight chambers where it was not possible to replenish the gas absorbed by the walls of the chamber, inhaled by the animals, etc. In the present experiments all the animals were exposed to a continuous flow of a controlled gas concentration.

The single-dose, air-tight chamber experiments give trustworthy figures only at very toxic concentrations where the animals die before the concentration is significantly diminished. The results of longer exposures are questionable because of the rapid drop in concentration. Unless otherwise noted, all the experiments reported in the literature cited below were single-dose experiments.

Horvath (7) found that rabbits and guinea pigs exposed to  $\text{NH}_3$  ranging in concentration from 0.25 per cent to 1.5 per cent by volume (2500 p.p.m. to 15,000 p.p.m.) developed acute and chronic lung lesions. Rabbits were less sensitive than guinea pigs which tolerated 0.15 per cent (1500 p.p.m.)  $\text{NH}_3$ . Habituation to  $\text{NH}_3$  gas was not observed so that Lehmann's conclusion that  $\text{NH}_3$  concentrations up to 0.5 per cent (5000 p.p.m.) is safe in factory air for "habitual" workmen is questioned.

<sup>1</sup> Director, Jamestown Municipal Laboratory, Jamestown, New York and Director, Chautauqua County Laboratory, Dunkirk, New York.

Ireland and others (14, p. 307-355) classified  $\text{Cl}_2$  gas as a lung irritant. High concentrations had an instantaneous effect on the respiratory tract and eyes. They found that a half hour exposure to a concentration of 800 to 900 p.p.m. resulted in rapid acute death to about 50 per cent of the dogs exposed. These experimenters used a continuous flow of gas but had some difficulty in controlling their concentrations. They present a very thorough treatment of the symptomatology of chlorine gas poisoning. Injury to the tissues was confined to the respiratory tract, the principal symptoms on autopsy being edema in the lungs and necrosis of the bronchial epithelium. The reader is referred to this exhaustive work on war gases for a review of the literature on the effect of both  $\text{Cl}_2$  and  $\text{HCN}$  gases on higher animals.

Skljanskaja and Rappaport (13) observed catarrh-like changes in the upper respiratory tract, hemorrhages and emphysema in the lungs of rabbits exposed to a  $\text{Cl}_2$  concentration of 0.002 to 0.005 mg./l. of air (about 0.7 to 1.7 p.p.m.). They state that the action on the respiratory tract is to make it supersensitive to infection, especially to pneumonia. In two cases macerated embryos were observed in the abdominal cavity and the implications to human pathology are stressed.

Ireland and others (14, p. 407) note that  $\text{HCN}$  gas is not a lung irritant or lachrymator, but acts only after absorption in the circulation.  $\text{HCN}$  causes pulmonary stimulation followed by paralysis, and death is caused by failure of the respiratory functions. Symptoms in dogs included muscular weakness, loss of coordination, labored breathing, convulsions, and irregular respiration. At high concentrations (0.5 mg./l., about 450 p.p.m.) death occurred in five minutes. At low concentrations the symptoms appear less rapidly. They observed no delayed symptoms. Recovery always occurred if the animal survived two hours. They state that a man can exist without serious injury if exposed to 1 to 2000 (500 p.p.m.)  $\text{HCN}$  for one and one-half minutes, this being lethal for the dog.

Barcroft (2) gives  $\text{HCN}$  concentration-time curves for goats, monkeys, rabbits, rats, cats, dogs, guinea pigs, mice, fowls, canaries, and pigeons. He found the guinea pig the most tolerant of small quadrupeds, dogs the most sensitive. Canaries and pigeons were the most sensitive of all animals tested. When a dog (12 kg.) and a man (70 kg.) were exposed to  $\text{HCN}$  1 to 2000 (500 p.p.m.), the dog became unconscious in 1 minute and 33 seconds. The dog was removed from the chamber and eventually survived. The man also came out of the chamber having felt no symptoms, but from five to ten minutes later there was a momentary feeling of nausea and difficulty was experienced in concentrating during conversation. Barcroft states that there is a great stimulation of the respiratory center followed by inhibition. Larger animals as a rule were more resistant, but there were exceptions. The extreme sensitivity of dogs may be due, he states, to the fact that the dog inhales more air in proportion to its weight than other

animals. Canaries died in one minute when exposed to HCN 1 to 5000 (200 p.p.m.), while monkeys lived 25 minutes when exposed to this concentration. The concentration of HCN gas that killed a canary and made a pigeon vomit in two minutes was about 100 p.p.m. The canary and pigeon are suggested as biological indicators when unknown concentrations are encountered.

Barcroft's experiments were in closed chambers and he did not replenish the HCN lost by absorption and inhalation. He found a 22 per cent reduction of an initial concentration of about 190 p.p.m. in 38 minutes. His high concentration experiments, however, in which he obtained kills in less than five minutes are of considerable interest and value in connection with our study. The following table is based on Barcroft's Table II. His "lethal time" is time to kill 50 per cent of the exposed animals.

Animal	Lethal time of exposure to a concentration of 1.0 mg./l.—830 p.p.m.
	(min.)
Dog	0.8
Mouse	1.0
Cat	1.0
Rabbit	1.0
Rat	2.0
Guinea pig	2.0
Goat	3.0
Monkey	3.5

Drinker (4) reports a case of temporary cyanide poisoning of two workmen wearing effective gas masks in a concentration of 2 per cent HCN gas (20,000 p.p.m.) for eight to ten minutes. The poisoning was believed to be due to absorption through the skin.

Gettler and Blaine (5) describe analytical methods for the detection of cyanide poisoning.

Recently Carpenter and Moore (3) have studied the absorption of cyanide by insects. The amount absorbed varied with the species. Insects which are generally known to be difficult to kill were found to absorb smaller quantities of HCN than those species which are easily killed.

Young and Busbey (17) have compiled a bibliography of cyanide compounds used as insecticides.

Arkhangelsky (1) tested  $H_2S$  as an insecticide against different species of insects including the San José scale (*Aspidiotus perniciosus*), plant lice, and stored-grain insects. The concentrations of the gas ranged from 4 to 8 per cent (about 40,000 to 80,000 p.p.m.), and the periods of exposure

from one-half hour to 24 hours. In all cases, he reports that good results were obtained.

Yant (16), in a comprehensive review of  $\text{H}_2\text{S}$  as an industrial poison, states that  $\text{H}_2\text{S}$  occurs more widely, is more toxic, and constitutes a greater hazard than is generally realized. He states that the toxicity of  $\text{H}_2\text{S}$  is comparable to  $\text{HCN}$ . Two distinct types of poisoning are recognized—subacute and acute. The threshold concentration of acute poisoning is fixed at 0.07 per cent by volume (700 p.p.m.). Subacute poisoning produces direct irritating action on the tissues causing conjunctivitis and respiratory irritation. Acute poisoning is the result of action on the nervous system produced by the absorption and presence of  $\text{H}_2\text{S}$  in the blood. There are no warning symptoms. Unconsciousness and failure of respiration occur in a few seconds after exposure, followed in five to ten minutes by cardiac failure. Death is due primarily to asphyxia. He reports no abnormal combinations with hemoglobin, no evidence of cumulative effect or chronic type of poisoning. The gas is rapidly oxidized in the blood to form non-toxic compounds, ultimately sulphates. Although the odor of  $\text{H}_2\text{S}$  may be detected at a high dilution (1 part in 10 million parts of air), fatigue to the sense of smell occurs rapidly and cannot be depended upon for warning against dangerous concentrations. High concentrations of  $\text{H}_2\text{S}$  (100 per cent) have a surface action on the skin causing discoloration and erythema but exposure for one hour did not produce nervous symptoms.

The literature on  $\text{SO}_2$  has been reviewed in our previous publication (15).

#### APPARATUS

The apparatus used for maintaining constant concentration of the gas has been described by Setterstrom and Zimmerman (12), and by McCallan and Setterstrom (9). It is the same apparatus as described in our previous paper (15) on the effect of sulphur dioxide on animals. Special precaution was taken to prevent  $\text{HCN}$  condensation and the apparatus in this case was kept at a temperature of  $27^\circ\text{C}$ .

#### DESIGN OF EXPERIMENTS

House flies (*Musca domestica* L.) were selected as the invertebrate test material while rats and mice were selected for the vertebrate material. The animals were exposed to different concentrations of ammonia, chlorine, hydrogen cyanide, hydrogen sulphide, and sulphur dioxide for different lengths of time. In the case of the flies, the cages were removed to the insectary and mortality counts were made 24 hours after exposure. The rats and mice that survived the treatments were removed to a favorable environment for observation of possible delayed effects of the gas. Gas concentrations tested were 16, 63, 250, and 1000 p.p.m. Periods of exposure were 1, 4, 15, 60, 240, and 960 minutes.



The experiments were performed during the months of September, October, November, December, and January. The tests were begun at 5:30 p.m. in order to reduce temperature and light fluctuation to a minimum. The mean temperature during the gas treatments was  $73.3 \pm 3.3^{\circ}\text{F.}$ , while the mean relative humidity was  $74.6 \pm 8.8$  per cent.

Results are presented in the form of time-mortality curves, expressing per cent kill as a probability function and time logarithmically in order to obtain a straight line relationship. This method of expression is simplified by the use of logarithmic-probability paper.<sup>2</sup>

In comparing the effects of the various concentrations of the various gases, time till death of half the animals  $\text{LT}_{50}$  (9, p. 326) was used. This is accepted as the most precise and reproducible point on a toxicity curve. The  $\text{LT}_{50}$  does not, however, give any indication of the slope of a curve. A comparison of  $\text{LT}_{50}$ s represents comparative toxicities over the whole range of per cent kill only if the toxicity curves are essentially parallel. In comparing the toxicity of gases with non-parallel time-mortality curves, one gas, for example, may be more effective than another at  $\text{LT}_{50}$  but less at  $\text{LT}_{80}$ .

## HOUSE FLIES

### REARING METHODS

The flies were reared in general according to methods set forth by the National Association of Insecticide and Disinfectant Manufacturers (10). Certain modifications were made and are described below.

Eggs were collected from the absorbent cotton in the feeding dishes from the rearing cages and placed in water. The egg clumps were broken up and the eggs that floated were removed by decantation. Advantage was taken of the fact that sterile eggs float and fertile eggs sink. A suspension of fertile eggs was made by shaking them in water in a flask. The egg suspension was poured into a graduated centrifuge tube and allowed to settle until 1 cc. of eggs had settled out. One cc. of eggs was added to each battery jar half full of medium. The eggs were covered with one-half inch of the medium and the mouth of the jar was protected by a single layer of cheesecloth.

A modified Richardson (11) medium was used. It consisted of a one-pound mixture containing one-third part alfalfa meal and two-thirds part wheat bran to which was added one ounce of yeast, and 3 cc. of malt syrup in one liter of water. The medium was allowed to stand for 12 hours before using.

Larvae pupated in the upper half inch of the medium in the battery jars in seven days and the pupae were removed nine days after the eggs were added to the jar. The pupae were cleaned and dried by a winnowing

<sup>2</sup> The Codex Company, Norwood, Massachusetts.

process which consisted in allowing them to fall in a current of air produced by an electric fan.

The flies were apportioned to the cages by weighing 5-g. lots of pupae. It was found that this amount would yield approximately 250 flies. Wire cages (10×9×18 in.) were used. There was a sliding door at one end of the cage for introduction of pupae and food and for removal of the flies.

The flies in each cage were fed daily 60 cc. of a half and half mixture of milk and water. Tin dishes were used. A square of absorbent cotton was placed in the dish and the solution poured over the cotton.

The flies were reared in an insectary that is air-conditioned by means of a Carrier unit. The air is washed before it enters the room and recirculated through the system. Air from the outside is drawn in and heated before it is circulated. The conditions found most satisfactory for the rearing room were a temperature of 81° F. and a relative humidity of 50 per cent. The temperature was maintained within one degree and the relative humidity varied only slightly ( $\pm 5\%$ ).

#### EXPERIMENTAL PROCEDURE

The flies were exposed in cages to a given concentration of the gas. Generally, a given experiment was performed on one day with a set of flies for each period of exposure to a given concentration of gas. Another experiment at a different concentration on a different day would involve a different set of flies and thus introduce a day-to-day variation. Replicates of a given concentration on the same day and on different days were made in some instances. Immediately after exposure to the gas the cage containing the flies was removed from the cabinet and examined for any evidence of stunning effect ("knockdown") on the flies. After this observation was completed the cage containing the flies was removed to the insectary.

The highest concentration of a given gas was tested first, then the next highest, and so on until a concentration was reached that would not produce an appreciable kill in 16 hours, when the series was discontinued.

#### EXPERIMENTAL RESULTS

NH<sub>3</sub> was found to be the least toxic of the five gases tested, so that only two exposures were made at the same concentration. The kill obtained with 1000 p.p.m. was 6 per cent in 4 hours, and 39.4 per cent in 16 hours. The results obtained with Cl<sub>2</sub>, HCN, H<sub>2</sub>S, and SO<sub>2</sub> are shown graphically in Figures 1 and 4. It will be noted in Figure 4 that the mortality falls rapidly with all the gases except HCN when concentrations below 1000 p.p.m. are used. Rats and mice, it will be observed, are more resistant to this gas than are house flies. This suggests the possibility that a low uniform concentration of HCN might be maintained in fumigation with little or no hazard to warm-blooded animals and still be effective on insects.

HCN was the only gas that produced a marked knockdown. At a concentration of 1000 p.p.m. it produced 100 per cent knockdown in one and one-third minutes. This compares with estimates of 50 per cent for Cl<sub>2</sub> and H<sub>2</sub>S at the same concentration for 10 minutes, while SO<sub>2</sub> and NH<sub>3</sub> showed only 60percent and 30percent knockdown respectively in 25minutes.

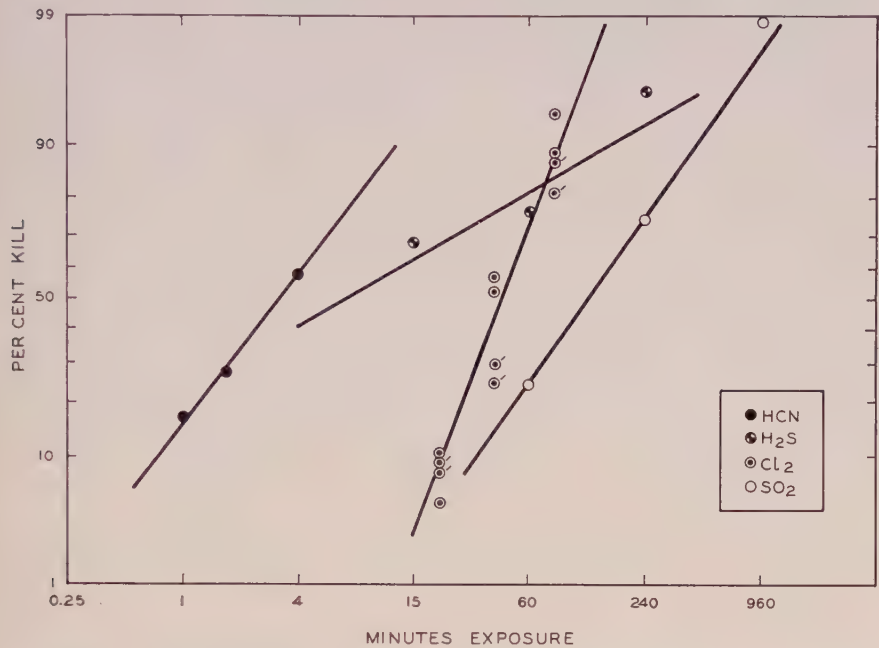


FIGURE 1. Time-mortality toxicity curves for house flies exposed to 1000 p.p.m. of HCN, H<sub>2</sub>S, Cl<sub>2</sub>, and SO<sub>2</sub>. Replicates of Cl<sub>2</sub> on a different day indicated by prime (') marks.

There was good agreement between duplicates run on the same day, but comparison of duplicates run on different days showed the usual day-to-day variation (compare tests 1 and 2, Table I). The natural mortality was less than 3 per cent.

TABLE I  
RESULTS OBTAINED WITH Cl<sub>2</sub> AT 1000 P.P.M. USING AVERAGE 5-DAY-OLD  
ADULT HOUSE FLIES  
(Duplicate Tests)

Test	% Mortality at given time intervals in minutes				
	5	10	20	40	80
1	3.2	0	8.3	24.4	81.9
	0.6	0	7.4	29.8	87.5
2	3.7	1.5	9.2	56.6	88.2
	4.0	2.7	4.6	52.7	93.9

The comparative susceptibility of flies of different ages is shown in Table II. Flies exposed to  $\text{SO}_2$  1000 p.p.m. for one hour showed a kill of approximately 22 per cent with no significant difference between the age

TABLE II  
EFFECT OF  $\text{SO}_2$ ,  $\text{Cl}_2$ ,  $\text{H}_2\text{S}$ , AND  $\text{HCN}$  1000 P.P.M. ON HOUSE FLIES AT DIFFERENT AGES

Gas	Time exposed	% Mortality at different average ages in days		
		1-2	4	7-8
$\text{SO}_2$	1 hr.	21.1	21.8	22.8
$\text{Cl}_2$	1 hr.	76.7	91.2	88.5
$\text{H}_2\text{S}$	1 hr.	86.9	75.3	58.4
$\text{HCN}$	4 min.	80.0	64.2	40.4

groups. With  $\text{HCN}$  and  $\text{H}_2\text{S}$  at the same concentration the 7- to 8-day-old flies were more resistant than 1- to 2- or 4-day-old flies, while with  $\text{Cl}_2$ , 1- to 2-day-old flies were the most resistant.

Flies exposed to  $\text{Cl}_2$  1000 p.p.m. for 10 minutes in the cabinet (72 cu. ft.) as compared with flies exposed simultaneously in small wire cages (0.9 cu. ft. each) in the same cabinet showed an average mortality of approximately 35 per cent for the flies released in the cabinet as compared with approximately 4 per cent for the flies exposed in small cages. This difference in kill is believed to be due to the greater activity on the part of the flies that had the whole range of the cabinet because of its greater capacity than the individual cages. Jongbloed and Wiersma (8) have shown that the oxygen consumption of insects in flight may be 48 times that for the same insect when at rest.

## RATS AND MICE

### EXPERIMENTAL PROCEDURE

Young vigorous mature male and female rats and mice were used in these experiments. Eight rats and four mice were used at each effective concentration of each gas. All were exposed for 16 hours or until death. Rats and mice of the same age as the treated individuals were used as controls. As the exposures were of relatively short duration, no record of weights was made. The animals that survived the treatments and the controls were kept under observation about 5 months, until March 7, when autopsies were made of representative individuals. The rats and mice were fed "complete ration" dog pellets<sup>3</sup> daily in excess of what they would accept. A ration of cod liver oil at the rate of 1 cc. per rat and 0.25 cc. per mouse on cabbage leaves was furnished twice a week. Water was supplied by means of drop fountains.

<sup>3</sup> The manufacturer's analysis lists not less than 24 per cent protein, not less than 3 per cent fat, not more than 5 per cent fiber, not less than 50 per cent carbohydrates.



The animals were exposed to the gas in the cabinet in wire cages. Careful observations were made at frequent intervals and signs and symptoms noted. Upon death of an animal the body was removed to a 10° C. cold room to await autopsy in the few cases in which immediate autopsy was not possible. Viscera were preserved in formalin or in Bouin's solution.

The various concentrations of a given gas were tested in descending order until a concentration was reached that would not produce an appreciable kill in 16 hours, when the series was discontinued. For this reason the lowest concentrations tested were: ammonia—1000 p.p.m.; chlorine—16 p.p.m.; hydrogen cyanide—16 p.p.m.; hydrogen sulphide—16 p.p.m.; and sulphur dioxide—250 p.p.m.

EXPERIMENTAL RESULTS

Time-mortality curves on logarithmic-probability coordinates are presented in Figure 2 for rats and in Figure 3 for mice. The times-till-death of half the animals as obtained from these curves are plotted against the concentration in Figure 4 which also presents similar curves for the flies. To make intercomparisons of the three organisms easier, LT50s are also given in Table III.

TABLE III  
TIME TILL 50 PER CENT MORTALITY OF GAS-TREATED ANIMALS  
(IN MINUTES)

Gas	Animal	Concentration, p.p.m.			
		1000	250	63	16
NH <sub>3</sub>	Flies	>960			
	Mice	>960			
	Rats	>960			
Cl <sub>2</sub>	Flies	45	240	840	>960
	Mice	28	440	>960	
	Rats	53	440	>960	
HCN	Flies	3.3	<8	8.2	48
	Mice	1.2	5.1	66	>960
	Rats	1.4	8.7	40	>960
H <sub>2</sub> S	Flies	7	>960		
	Mice	18	410	804	>960
	Rats	14	>960	>960	>960
SO <sub>2</sub>	Flies	120	720		
	Mice	132	786	>960	
	Rats	910	>960		

Signs and symptoms which developed during exposure to the gas as well as pathological symptoms revealed on autopsy are presented in detail under the headings of the various gases.

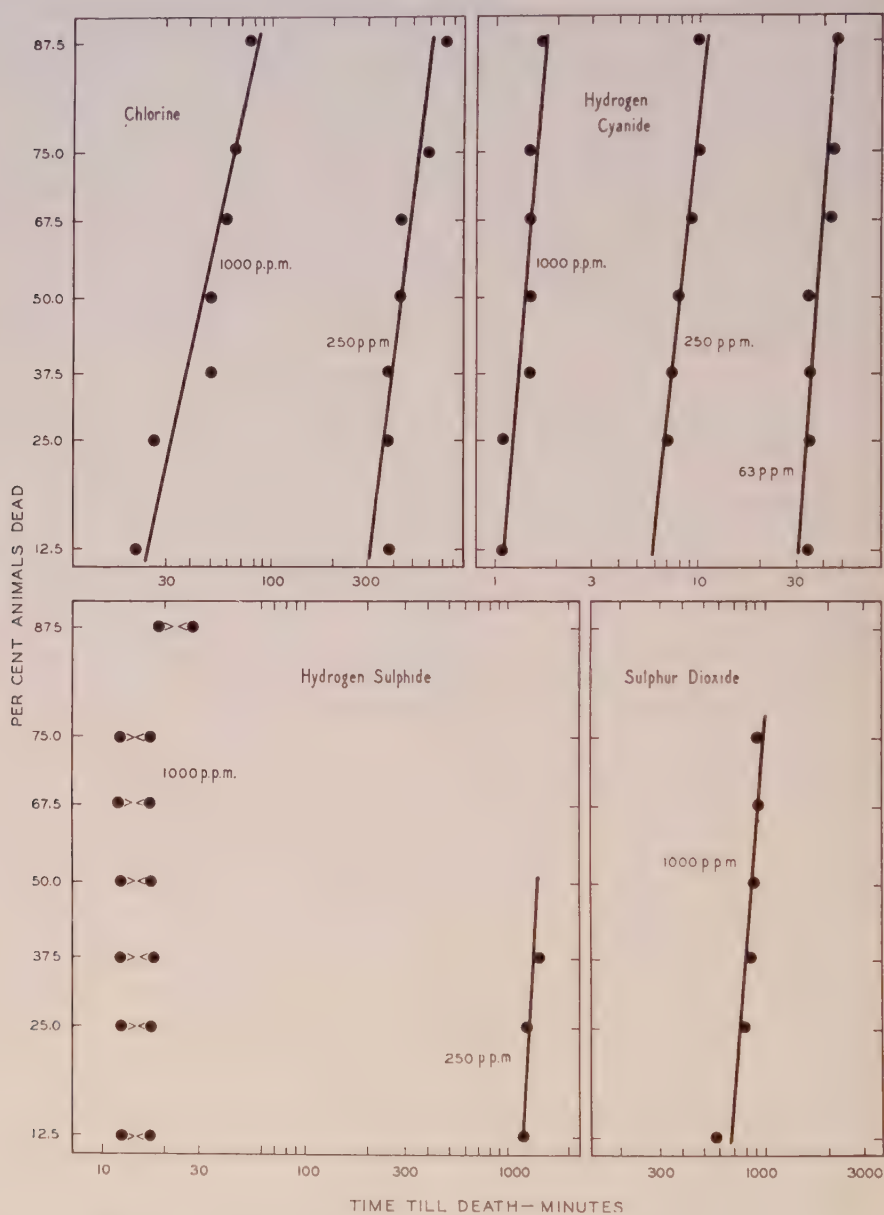


FIGURE. 2. Time-mortality toxicity curves for rats exposed to chlorine, hydrogen cyanide, hydrogen sulphide, and sulphur dioxide.

*Effect of NH<sub>3</sub> on Rats and Mice*

The animals exposed to 1000 p.p.m. of NH<sub>3</sub> were not noticeably affected and even continued to eat undisturbed. They began to rub their noses with

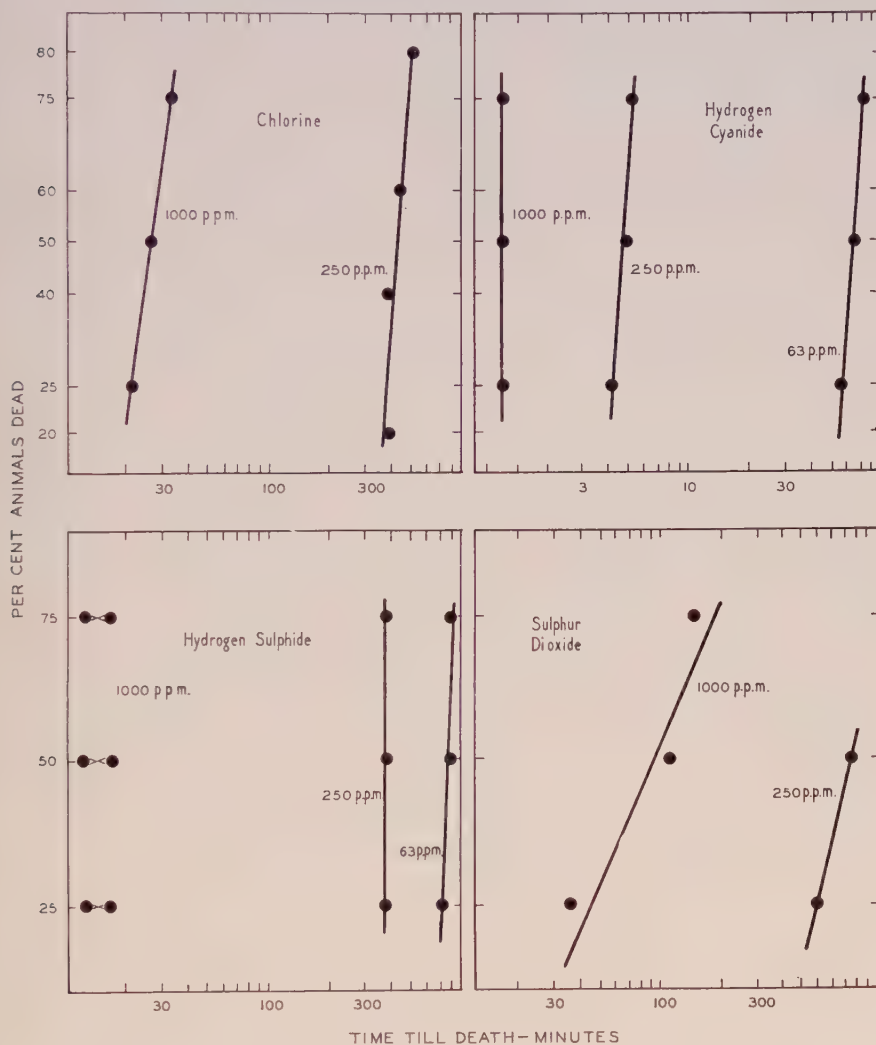


FIGURE 3. Time-mortality toxicity curves for mice exposed to chlorine, hydrogen cyanide, hydrogen sulphide, and sulphur dioxide.

their fore feet at the end of a half hour. They continued to eat occasionally and to climb around the cages. Several began digging into the bedding at the end of the first hour and two older individuals showed some evidences

of a slight dyspnea. At the end of the exposure (16 hrs.) the eyes of all the rats and mice were bright with little or no evidence of lachrymation. The mice seemed even less disturbed by the gas than the rats. They remained quiet, huddled together. Occasionally an individual would clean its fur.

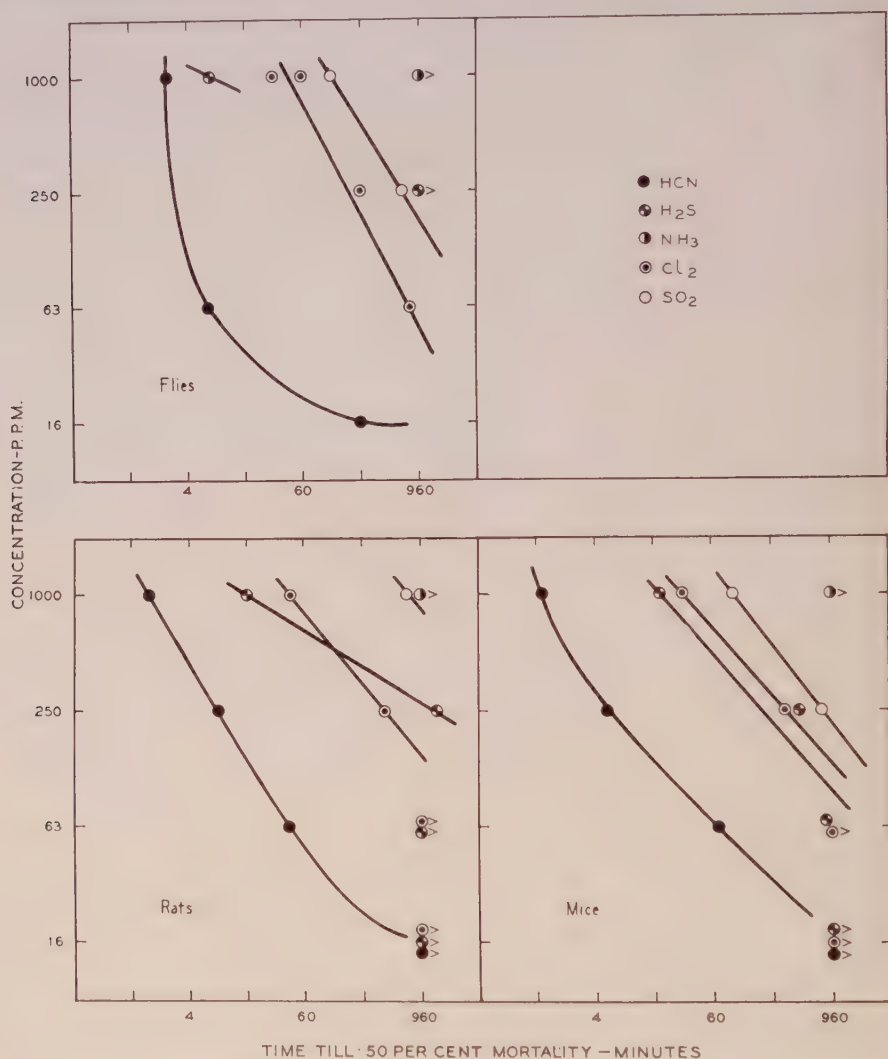


FIGURE 4. Time-concentration toxicity curves at 50% mortality for house flies, rats, and mice exposed to HCN, H<sub>2</sub>S, NH<sub>3</sub>, Cl<sub>2</sub>, and SO<sub>2</sub>. (See also Table III.)

Two rats killed for autopsy March 7, 1940, showed all organs natural. The findings for a pregnant female mouse killed on the above date showed a few fresh hemorrhages (traumatic) of the pleural cavity. Another mouse showed all organs natural throughout.



Typical gross findings at autopsy for a rat which died 12 hours after exposure to the gas appear in Table IV.

TABLE IV  
TYPICAL GROSS FINDINGS AT AUTOPSY OF RATS AND MICE EXPOSED  
TO 1000 P.P.M. OF  $\text{NH}_3$  AND OF  $\text{SO}_2$

Organs	$\text{NH}_3$	$\text{SO}_2$
	Rat which died 12 hrs. after exposure	Rats and mice which died in the gas
Brain	Slightly congested.	Slightly to moderately congested.
Trachea	Not reddened.	Not reddened.
Lungs	Two-thirds distended, many large hemorrhages, cherry-red, waxy, cut surface foamy.	Fairly well collapsed, color pink, slightly emphysemic, no massive hemorrhages, cut surfaces wet but not foamy. A few small hemorrhages.
Heart	Much distended.	Acute dilatation, right side.
Liver	Congested.	Moderately congested.
Gall bladder	Not distended.	Not distended (occasionally distended in mice).
Stomach	Moderately distended, few hemorrhages.	Definitely distended, many scattered minute hemorrhages.
Intestines	Large intestine partly distended.	Small intestine not distended, ascending colon greatly distended.
Adrenals	Pink.	Natural.
Kidneys	Congested.	Moderately congested.
Peritoneal surfaces	Not remarkable.	Bright pink, fading after section.

*Effect of  $\text{Cl}_2$  on Rats and Mice*

The rats and mice exposed to 1000 p.p.m. of  $\text{Cl}_2$  showed very little initial excitement. Moderate dyspnea, foamy secretion at the nostrils, and marked lachrymation were noticed. The first rat died in 20 minutes, showing no additional signs except prostration and terminal convulsions. All were dead in 1.7 hours. A mouse was dead in 21 minutes and all were dead in 50 minutes from the time they were placed in the cabinet. The signs in the mice were similar.

During the first hour the rats continued to sniff  $\text{Cl}_2$  when exposed to 250 p.p.m. They showed lachrymation and at 4 hours dyspnea. At the end of 16 hours all the rats of this series were dead. The first died in 6.4 hours, and the last in 16 hours. The mice showed lachrymation but very little other change during the first hour of exposure, thereafter dyspnea and terminal prostration and convulsions. All the mice of this series were dead in 8.4 hours.

Rats and mice exposed to  $\text{Cl}_2$  63 p.p.m. exhibited less distress than those exposed to 250 p.p.m. Two rats were killed for autopsy March 7, 1940. One individual showed a few old adhesions in the lungs, and all other organs natural. The other individual showed all organs natural throughout.

Typical gross findings for rats and mice which died in the gas or were autopsied immediately after exposure, appear in Table V.

TABLE V  
TYPICAL GROSS FINDINGS AT AUTOPSY OF RATS AND MICE WHICH DIED DURING EXPOSURE TO  $\text{Cl}_2$  OR WERE SACRIFICED IMMEDIATELY AFTER GAS TREATMENT

Organs	Concentration of $\text{Cl}_2$ gas			
	1000 p.p.m.	250 p.p.m.	63 p.p.m.	Rats*
Brain	Slightly congested.	Slightly congested.	Slightly congested.	Slightly congested.
Trachea	Not reddened.	Not reddened.	Not reddened.	Not reddened.
Lungs	Distended filling cavity, pale waxy, cut surfaces foamy.	Partly collapsed and hemorrhagic.	Deep black-red, cut surfaces drip blood.	Distended, pink, very rare punctate hemorrhages, cut surfaces wet and foamy.
Heart	Greatly distended on right side, atria distended.	In systole or moderately distended.	In systole.	Right side distended.
Liver	Congested.	Congested.	Twice natural size, waxy pale, nutmeg.	Pale to dark red in color.
Gall bladder	Not distended.	Not distended.	Not distended.	Not distended.
Stomach	Moderately to greatly distended, few small hemorrhages.	Greatly distended, very rare small hemorrhages.	Moderately distended.	Distended, very rare punctate hemorrhages.
Intestines	Large and small, partly distended.	Large and small, moderately distended.	Slightly distended.	Small intestine, moderately distended; colon moderately distended.
Adrenals	Pink.	Pink.	Pale.	Pale to natural pink in color.
Kidneys	Congested.	Congested.	Pale.	Pale to dark red in color.

\* Autopsied immediately after exposure.

*Effect of HCN on Rats and Mice*

The eight rats of the 1000 p.p.m. experiment were exposed in groups of two. They exhibited characteristic signs in a definite sequence. The rats became wildly excited, leaping and climbing about the wire cages in from 3 to 23 seconds. They were down on their backs, but still breathing in 25 seconds. They were comatose in from 38 to 62 seconds. Last respiration occurred in from 67 to 100 seconds from the time they were placed in the cabinet. Four mice which were exposed to the gas showed similar signs as to those of the rats but in somewhat shorter time intervals. The wildly excited stage occurred in 8 seconds, they were down on their backs in 25 to 50 seconds, and last respiration occurred in 85 seconds from the time they were placed in the cabinet.

The eight rats used at 250 p.p.m. were also exposed in groups of two. The signs exhibited were similar to those previously described for 1000 p.p.m. although the onset was delayed. The period of wild excitement occurred in from 22 to 38 seconds. They were down on their backs but still breathing in from 55 to 80 seconds. The first rat was dead in 6.7 minutes and the last one in 10.4 minutes. Four mice exhibited signs similar to the rats. The period of excitement occurred in 45 seconds followed by prostration in 88 seconds. They were comatose in 100 seconds and last respiration occurred 6 minutes from the time they were exposed to the gas.

The rats introduced into the cabinet containing HCN 63 p.p.m. showed slightly increased activity with loss of coordination of muscles within 30 seconds. After 9 minutes the respiration was deep and slow (20 per min.). Occasional tremors occurred in the next phase which lasted 2 minutes. Short, shallow respiration (72 per min.) continued for 11 minutes, decreasing in rate to about 27 per minute at 15 to 22 minutes. During this time the breathing was by means of abdominal muscles and diaphragm followed by a slightly lagging chest movement. At 22 to 30 minutes, respiration was by abdominal muscles only, followed by convulsions and chest respiration. The first animal died at 34 minutes', the last at 82 minutes' exposure. The signs for the mice were found to be somewhat similar to those found for rats but the speed of mortality was found to be greater for the rats than for the mice. Thus it was found to require 48 minutes to kill six rats, while all of the mice were alive at the end of this period. The first mouse of this series died in 57 minutes while the last mouse died in 3 hours and 35 minutes.

Both rats and mice exposed to HCN 16 p.p.m. showed no detectable evidence of distress throughout the period of exposure. They continued to eat and move about in a normal manner. Their eyes were bright and fur smooth at the end of the exposure. Two rats killed for autopsy March 7, 1940, showed all organs natural in one individual. The other individual

TABLE VI  
TYPICAL GROSS FINDINGS AT AUTOPSY OF RATS AND MICE WHICH DIED DURING EXPOSURE TO HCN

Organs	Concentration of HCN gas on both rats and mice		
	1000 p.p.m.	250 p.p.m.	63 p.p.m.
Brain Trachea Lungs	Natural. Natural. Well collapsed, dark pink, very rare minute pleural hemorrhages.	Slightly congested. Natural. Well collapsed, dark pink, a few scattered pleural hemorrhages up to 0.1 cm. in diameter.	Slightly congested. Natural. Well collapsed, usually orange-red but occasionally pink, a few scattered pleural hemorrhages up to 0.1 cm. in diameter.
Heart	Ventricles in systole, atria moderately distended. Right atrium beats upon stimulation.	Ventricles in systole, atria moderately distended. Beat upon stimulation.	Ventricles in systole, atria moderately distended. Does not beat upon stimulation by pricking with scissors.
Stomach	Very rare, very minute circular dark hemorrhages in wall.	Very rare, very minute circular dark hemorrhages in wall.	Very rare, minute circular dark hemorrhages in wall; occasionally somewhat larger hemorrhages; stomach is distended (in rats only).
Gall bladder	Not distended.	Not distended.	Not distended.
Intestines	Not distended.	Not distended, veins distended.	Not distended, veins distended.
Liver	No definite change.	Slightly cyanotic.	Slightly cyanotic.
Abdominal veins	No definite change.	Moderately distended.	Moderately distended.



showed pseudotuberculosis of both lungs. A mouse killed for autopsy on the above date showed all organs natural throughout.

Typical gross findings of rats and mice which died in the gas are shown in Table VI.

#### *Effect of H<sub>2</sub>S on Rats and Mice*

The rats were very active, climbing around the cages and jumping about during the first five minutes of exposure to 1000 p.p.m. At the end of five minutes lack of muscular coordination was evident; the animals staggered and climbed about wildly, then relapsed into a coma only to begin their violent uncoordinated movements a few minutes later. All individuals were prostrated in 11 minutes, partly on their backs with their abdomens swollen. All the rats were dead in from 29 to 37 minutes. The mice were very active during the first few minutes of exposure to the gas. There was marked lachrymation. The first death occurred in 18 minutes and all were dead in 20 minutes.

During the first 25 minutes of exposure to H<sub>2</sub>S 250 p.p.m. the rats were restless and continued climbing about the cages, sniffing and rubbing their noses with their fore feet. They became quiet in 25 minutes and manifested little discomfort. One individual was observed eating after 2 hours. The first rat died at the end of 17.9 hours. Three were dead at the end of 22.9 hours when the experiment was discontinued. The mice exhibited symptoms very similar to the rats during the first hour of exposure. At the end of 2 hours they were gasping moderately and their abdomens were distended. The first three individuals died in 6.9 hours, and all were dead at the end of 7 hours. Two rats killed for autopsy March 7, 1940, showed all organs natural.

The rats were not markedly affected during the first hour of exposure to H<sub>2</sub>S 63 p.p.m. but continued to eat and drink undisturbed. Even after 16 hours' exposure the seven surviving rats appeared to be in fair condition, although lethargic and breathing heavily. The mice showed similar but more marked signs than the rats. The first mouse died in 57 minutes. After 16 hours' exposure the one surviving mouse continued crawling around the cage. This individual was found dead 23 hours later.

On initial exposure to 16 p.p.m. the animals showed some slight restlessness but quickly became habituated, and showed no other abnormal symptoms at this concentration.

A mouse killed for autopsy March 7, 1940, which had been exposed to H<sub>2</sub>S 16 p.p.m. for 16 hours showed all organs natural throughout.

Typical gross findings at autopsy for rats and mice which were exposed to H<sub>2</sub>S and died in the gas appear in Table VII.

TABLE VII  
TYPICAL GROSS FINDINGS AT AUTOPSY OF RATS AND MICE WHICH DIED DURING EXPOSURE TO H<sub>2</sub>S

Organs	Concentration of H <sub>2</sub> S gas					
	1000 p.p.m.		250 p.p.m.		63 p.p.m.	
	Rats	Mice	Rats	Mice	Rats	Mice
Brain	Very slightly congested.			Slightly congested.		
Trachea	Natural.	Natural.		Natural.		
Lungs	Well collapsed, dark pink, cut surface wet, rare small hemorrhages	Same as rats.	Partly distended, extremely hemorrhagic.	Massive hemorrhages of all lobes,	One-half collapsed. Many small hemorrhages.	Natural. Deep red, apparently massive hemorrhage.
Heart	In systole, auria dilated, blood fluid.	Same as rats.	Distended.	Moderate dilatation of right side.	Moderate dilatation of right side.	Natural. Deep red, apparently massive hemorrhage.
Liver	Much congested.	Same as rats.	Congested.	Moderately enlarged, very pale, lobules not exaggerated.	Moderately dark red.	Moderately dilated. Pale, nutmeg, large.
Gall bladder	Not distended.	Same as rats.	Not distended.	Definitely but moderately distended.	Not distended.	Not distended to moderately distended.
Stomach	Moderately to much distended, few small hemorrhages.	Same as rats.	Distended, few small hemorrhages.	Definitely but moderately distended, rare minute hemorrhages.	Definitely but moderately distended, moderate number of small hemorrhages.	Moderately distended, few hemorrhages of moderate size.
Intestines	Natural or with a few small hemorrhages.	Same as rats.	Large, partly distended.	Small intestine slightly distended.	Caecum, moderately distended.	Duodenum dilated.
Adrenals	Natural, pink.	Same as rats.	Pink.	Pale.	Natural.	Natural.
Kidneys	Much congested.	Moderately congested.	Congested.	Pale.	Moderately dark red.	Pale.

*Effect of SO<sub>2</sub> on Rats and Mice*

The rats exposed to 1000 p.p.m. were very uneasy, digging into the bedding on initial exposure. At the end of a half hour they were gasping, although one individual was observed eating. Lachrymation was evident at this time. All the animals were still moderately active at the end of the first hour. Death of the first rat occurred in 9.5 hours. At the end of 16 hours lachrymation was notable and moderate dyspnea was present in the two remaining animals. One of these rats died after 16.5 hours, and the other 48 hours from the beginning of the exposure.

The mice were less active than the rats. The first death occurred in 37 minutes, the last death in 3.3 hours.

At 250 p.p.m. the rats coughed and sneezed on initial exposure, and were restlessly active during the first hour. No rat deaths occurred at this concentration, but at the end of the 16-hour exposure the animals were markedly dyspnotic, their eyes were incrustated and their nostrils filled with bloody mucus.

The mice exhibited similar but more marked symptoms. Two mice died during the exposure, one in 10 hours, the other in 14.9 hours.

Typical gross findings at autopsy for rats and mice which died in the gas appear in Table IV.

## DISCUSSION AND CONCLUSIONS

House flies, mice, and rats were exposed to a continuous flow of NH<sub>3</sub>, Cl<sub>2</sub>, HCN, H<sub>2</sub>S, and SO<sub>2</sub> gases under controlled conditions at concentrations of 16, 63, 250, and 1000 p.p.m. for periods up to 16 hours.

Toxicity curves are presented for each of the organisms at the various concentrations for the various gases; visible signs and symptoms are described; vertebrate pathological symptoms on autopsy are summarized.

The order of decreasing toxicity of the gases to each of the three organisms tested was found to be HCN, H<sub>2</sub>S, Cl<sub>2</sub>, SO<sub>2</sub>, and NH<sub>3</sub>.

In house flies, age and cage size were found to be factors in susceptibility to the different gases.

During exposure to Cl<sub>2</sub> the rodents showed little initial excitement but early signs of irritation of eyes and nose and early and increasing signs of pulmonary edema. Except for terminal convulsions, there was comparatively little activity.

In HCN after an initial lag period the rodents showed a period of wild excitement and loss of muscular coordination followed by coma ending in a terminal convulsion. At progressively lower concentrations all these periods including the initial lag were progressively longer but still definite including the 63 p.p.m. level. However, at 16 p.p.m. the rodents showed no effect. It is of interest that at 63 p.p.m. the LT<sub>50</sub> was much greater for mice than for rats though the rats died later at the higher concentrations.

This recalls the observation in the earlier paper in this series (15) that mice were found to be much more resistant than guinea pigs at concentrations of  $\text{SO}_2$  of 150 p.p.m. and below, but were much more susceptible at 300 p.p.m. and 1000 p.p.m., indicating a difference in the slope of the dosage-time curves of the two species.

At high concentrations of  $\text{H}_2\text{S}$  there was violent activity and loss of muscular coordination very similar to that seen with  $\text{HCN}$ ; at progressively lower concentrations there was progressively less activity. In addition in the lower concentrations there was evidence of irritation of eyes and nose and of pulmonary edema increasing with time of exposure.

Exposure to  $\text{NH}_3$  caused only very slight irritation to eyes and nose.

With  $\text{SO}_2$  the signs qualitatively resembled those of  $\text{Cl}_2$ .

Study of the organs at death revealed that the rats exposed to  $\text{Cl}_2$  showed much edema and slight to moderate hemorrhage of the lungs while the mice showed somewhat less edema and more hemorrhage, the hemorrhage probably causing death in the smaller animal before the edema was fully developed. The heart was more dilated in rats than in mice.

The organs of the rodents dying in  $\text{HCN}$  showed no change of importance except a bright red color of the blood in some animals.

Examination of rodents exposed to 1000 p.p.m. of  $\text{H}_2\text{S}$  showed only slight to moderate change of heart and lungs while those exposed to 250 p.p.m. showed severe pulmonary edema and hemorrhage and definite dilatation of the heart.

In default of any rodents dying in  $\text{NH}_3$  the changes in the one dying later may be noted and are those of acute pulmonary edema.

In those rodents exposed to  $\text{SO}_2$  at 1000 p.p.m. in spite of the difference between the  $\text{LT}_{50}$  of the mice and that of the rats there was little difference in the appearance of the lungs, which were moderately edematous and showed few hemorrhages. The heart in both species was usually much dilated.

As for the other organs venous congestion was not great in any animal. In all, the stomachs were moderately dilated but without ulcers such as were described in the longer experiments with  $\text{SO}_2$  in an earlier paper (15).

Chlorine thus appears to produce the greatest amount of hemorrhage and edema of the lungs,  $\text{H}_2\text{S}$  produces these effects also as well as a toxic effect of another type which causes cerebral excitement.  $\text{HCN}$  produces a similar excitement but no striking gross pathologic change. The effects of  $\text{SO}_2$  and  $\text{NH}_3$  resemble those of chlorine.

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## INDUCED FORMATION OF A $\beta$ -GENTIOBIOSIDE IN TOMATO ROOTS

LAWRENCE P. MILLER

Recent work has shown that when certain non-naturally occurring chemicals are absorbed by various higher plants, glycoside formation takes place with the foreign chemical serving as the aglucon. Just as in the case of the natural glycosides the sugar component of these glycosides is not always *d*-glucose. Thus while  $\beta$ -2-chloroethyl-*d*-glucoside (4) is formed in *Gladiolus* corms from absorbed ethylene chlorohydrin (2), corms of the same variety form a  $\beta$ -gentiobioside if *o*-chlorophenol is the foreign aglucon furnished (9). Experiments in which tomato plants (*Lycopersicon esculentum* Mill.) were grown in the presence of chloral hydrate or ethylene chlorohydrin showed that glycosides are also readily formed in this species from introduced chemicals (6). The glycoside formed from ethylene chlorohydrin is not  $\beta$ -2-chloroethyl-*d*-glucoside, however, even though tubers of the closely related *Solanum tuberosum* L. had been found to form this glucoside from ethylene chlorohydrin (5) when treated with this chemical in order to break the rest period (1, 3). The chlorine content of the glycoside resulting from chloral hydrate was also lower than that required for a compound involving only one molecule of *d*-glucose. Since  $\beta$ -*o*-chlorophenyl-*d*-glucoside and its tetraacetate (7) and  $\beta$ -*o*-chlorophenyl-gentiobioside heptaacetate and heptapropionate (9) had recently been synthesized and were thus available as reference compounds, it was thought desirable to grow some tomato plants in the presence of *o*-chlorophenol and attempt to determine the nature of the glycoside resulting from this aglucon. Results obtained would be expected to aid in the characterization of the glycosides formed from the other aglucons studied, although, as the work with *gladiolus* corms has shown, the sugar component would not necessarily be expected to be the same with all three aglucons.

The glycoside formed in tomato roots from absorbed *o*-chlorophenol has been found to be  $\beta$ -*o*-chlorophenyl-gentiobioside through the preparation of the acetyl and propionyl derivatives which are shown to be identical with the corresponding synthetic compounds. The work on the characterization of the glycosides formed from chloral hydrate and ethylene chlorohydrin is not yet completed and will be published in a later paper.

Preliminary results, as shown in a previous note (8), indicate that the glycoside formed from chloral hydrate is also, in all probability, a  $\beta$ -gentiobioside.

## EXPERIMENTAL

TREATMENT OF GROWING TOMATO PLANTS WITH *o*-CHLOROPHENOL

The plants to be treated were grown in sand cultures supplied with a nutrient solution and the *o*-chlorophenol was added to the cultures in small amounts over a period of two to three weeks. By this procedure the growing plant was furnished with only a relatively small amount of *o*-chlorophenol at one time. Preliminary tests had shown that *o*-chlorophenol is quite toxic to tomato plants and the addition of too large a quantity at one time to the plants resulted in severe injury. The plants were started in good greenhouse soil and were transferred at the start of the experiments to sand in 5-inch earthenware pots, supplied with saucers, and the treatment was not started until the plants had become well established. The nutrient solution added was made up from the following stock solutions: 2 per cent  $\text{KNO}_3$ ; 4 per cent  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ; 2 per cent  $\text{KH}_2\text{PO}_4$ ; 2 per cent  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 5 per cent ferric tartrate. The equivalent of 10 cc. of each of the four major nutrients was added to each pot together with 0.8 cc. of the ferric tartrate solution at the start of the cultures, and this application was repeated in three or four weeks as required. Since the plants were grown in sand culture for a relatively short time only, it was not found necessary to add any of the minor elements. The plants were watered by adding tap water to the saucers, care being taken to avoid splashing and consequent loss of some of the nutrient or *o*-chlorophenol present.

In the first experiment 25 tomato plants of the Marglobe variety were transferred to sand cultures when three to four inches high. Nutrients were added the following day and again 25 days later. The addition of *o*-chlorophenol was started when the plants were 13 to 14 inches tall, 33 days after having been transferred to the sand cultures. The *o*-chlorophenol was added four to six times per week, each pot receiving 0.1 millimole dissolved in 25 cc. of water at each application for the first 12 applications after which the quantity added was doubled. A total of 2.8 millimoles of *o*-chlorophenol was added to each culture. The plants were sampled two days after the last addition of *o*-chlorophenol. The tops and roots were ground separately through a food grinder and the juice expressed by squeezing through cheesecloth. The expressed juices were centrifuged, then heated to 80° C. and again centrifuged. In order to obtain an estimate of the amount of  $\beta$ -*o*-chlorophenyl glycoside present, portions of the juices were subjected to the action of emulsin (50 mg. per 100 cc.) in a N/20 acetate buffer of pH 4.75 at 35° C. for about 20 hours. The *o*-chlorophenol liberated was determined by distilling the solutions to one-half their volume and titrating the *o*-chlorophenol in the distillate by the method previously described (9). The root juice was found to contain 0.89 millimole of a  $\beta$ -*o*-chlorophenyl glycoside per 100 cc. after correcting for the 0.08 millimole obtained in a sample carried through without the addition



of emulsin. No evidence was obtained for the presence of an appreciable quantity of a  $\beta$ -glycoside in the juice from the tops, and a subsequent attempt to obtain a crystalline acetyl derivative of such a glycoside from the tops was unsuccessful.

In the second series 48 plants of the Bonny Best variety were used. The cultures were handled in the same way as those of the first series. The addition of *o*-chlorophenol was started three weeks after the plants were transferred to the sand cultures and 0.2 millimole was added at each application until 3.0 millimoles had been added to each plant. The plants were sampled two days after the last addition of *o*-chlorophenol. The root juice was found to contain 0.73 millimole of a  $\beta$ -*o*-chlorophenyl glycoside while the amount of *o*-chlorophenol recovered from the juice from the tops (0.08 millimole per 100 cc.) was not increased by previous treatment with emulsin.

The glycoside formed in tomato roots from the absorbed *o*-chlorophenol was obtained in crystalline form as the acetyl and propionyl derivatives from both series. The isolation from the second series only is given in detail below.

#### ISOLATION OF $\beta$ -*o*-CHLOROPHENYL-GENTIOBIOSIDE AS THE HEPTA- ACETATE AND HEPTAPROPIONATE FROM TOMATO ROOTS

The method used for obtaining a partially purified preparation of the *o*-chlorophenyl glycoside, suitable for acetylation, was essentially the same as that previously employed with gladiolus corms (9). The expressed juice from the tomato roots and a further extract obtained by mixing the residue remaining after expression of the juice with distilled water and again squeezing through cheesecloth were treated with an excess of lead acetate, filtered, and the excess lead removed by hydrogen sulphide. After filtering off the lead sulphide the solution was concentrated under reduced pressure and transferred to a Kutscher and Stendel continuous extractor, in which it was extracted with ethyl acetate. The material taken out by the ethyl acetate collected as a white amorphous deposit on the bottom of the flask, and it was therefore necessary to change the flask at intervals to prevent undue heating and consequent darkening of this extract. After seven four-hour extraction periods the ethyl acetate solutions were combined (except that the first one was not used since it seemed to contain more impurities than the others) and washed three times with water. This aqueous extract was then evaporated to dryness *in vacuo* and 20 cc. of dry pyridine and 12 cc. of acetic anhydride added. The acetylation was allowed to proceed overnight at room temperature and the reaction mixture was then poured into 150 cc. of ice-water. After standing for several hours the solution was filtered and the precipitate washed with cold water. The product was dissolved in warm acetone and crystallized after the addition of an equal

volume of absolute ethanol. The crude product (1.48 g.) was recrystallized twice from absolute ethanol. Yield, 0.89 grams, melting point  $207.5^{\circ}$  to  $208.5^{\circ}$  (corr.),  $[\alpha]_D^{26} = -49.9^{\circ}$  ( $\text{CHCl}_3$ , concn., 3.365). These constants compare with a melting point of  $207.5^{\circ}$ – $208.5^{\circ}$  and  $[\alpha]_D^{24} = -49.7^{\circ}$  obtained for synthetic  $\beta$ -*o*-chlorophenyl-gentiobioside heptaacetate (9). A mixed melting point determination showed no depression.

*Analysis:* Theory for  $\beta$ -*o*-chlorophenyl-gentiobioside heptaacetate,  $\text{C}_{32}\text{H}_{39}\text{O}_{18}\text{Cl}$ : Cl, 4.75. Found: Cl, 4.74, 4.61.

The extraction with ethyl acetate was continued for four additional 18-hour periods. The ethyl acetate extracts were washed with distilled water, the aqueous solutions evaporated to dryness *in vacuo* and 20 cc. of dry pyridine and 12 cc. of propionic anhydride added. After standing at room temperature overnight the reaction mixture was poured into 150 cc. of ice-water, filtered, and the product recrystallized from acetone and absolute ethanol. After three recrystallizations from 95 per cent ethanol, 1.03 g. melting at  $178.5^{\circ}$  to  $179^{\circ}$  were obtained. Specific rotation was found to be  $[\alpha]_D^{26} = -38.1^{\circ}$  ( $\text{CHCl}_3$ , concn., 2.675 g.). Synthetic  $\beta$ -*o*-chlorophenyl-gentiobioside heptapropionate has a melting point of  $178.5^{\circ}$  to  $179^{\circ}$  and a specific rotation  $[\alpha]_D^{26} = -38.0^{\circ}$  (9). No depression in melting point was observed in a mixed melting point determination.

#### DISCUSSION

Gentiobiose has not previously been known to occur in tomato plants. While these experiments offer no evidence as to whether gentiobiose is ordinarily a constituent of the tomato, they show that gentiobiose can be formed by this species under certain conditions. Since a gentiobioside has also been obtained from gladiolus corms, it seems likely that this sugar is more widely distributed in plants than was previously supposed. It is possible, of course, that the formation of  $\beta$ -*o*-chlorophenyl-gentiobioside may occur in such a way that no free gentiobiose is present at any stage. Thus  $\beta$ -*o*-chlorophenyl-*d*-glucoside may be formed first and this glucoside condensed with another molecule of *d*-glucose to form  $\beta$ -*o*-chlorophenyl-gentiobioside.

#### SUMMARY

When tomato plants are grown in sand cultures to which *o*-chlorophenol has been added, a  $\beta$ -*o*-chlorophenyl glycoside is formed in the roots. This has been shown to be  $\beta$ -*o*-chlorophenyl-gentiobioside through the preparation of the acetyl and propionyl derivatives which were found to be identical with the corresponding synthetic compounds.

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## COMPARISON OF AGRONOMIC VALUE OF THE INSOLUBLE NITROGEN DERIVED FROM UREA-AMMONIA LIQUOR-37 AND OTHER SOURCES

M. M. McCool

There has been a demand for water-insoluble nitrogen for many years by the users of commercial mixed fertilizers. For various reasons the supply of some of the most popular sources has become limited and, in addition, their cost high. In order to meet this situation a process, by means of which insoluble nitrogen may be synthesized, has been developed by the Ammonia Department of E. I. du Pont de Nemours & Co., Inc., Wilmington, Delaware. According to the above department, upon the addition of formaldehyde to urea-ammonia liquor, it reacts to form soluble hexamethylenetetramine. The mixture, trade name Urea-Ammonia Liquor-37 (UAL-37), is shipped in insulated tank cars to the fertilizer mixing plants. Upon treating superphosphate with this liquor, all free ammonia reacts with the superphosphate. The hexamethylenetetramine undergoes decomposition. The formaldehyde produced reacts with the urea forming compounds of undetermined composition. These urea-formaldehyde compounds subsequently undergo polymerization and become water-insoluble.

In the preparation of mixed commercial fertilizers the required amounts of Urea-Ammonia Liquor-37, superphosphate, potash, and other materials are mixed in a closed fertilizer mixer. The water in the liquor is absorbed as water of hydration and there results a mixed fertilizer of excellent mechanical condition.

The above organization brought this product to our attention with the request that the agronomic value of the insoluble nitrogenous material formed under different conditions be ascertained.<sup>1</sup> Accordingly, a study has been made of the effect of different degrees of ammoniation, reaction of the fertilizer, time of storage, temperature of storage, and methods of formation on the availability of the synthetic insoluble nitrogen, as indicated by nitrate production in the soil and plant growth. In addition, its agronomic value in comparison with that of cottonseed meal, process tankage, and garbage tankage has been investigated by means of plant growth tests in a greenhouse.

### MATERIALS AND METHODS

The nitrogen content and the neutral permanganate number or the amount of water-insoluble organic nitrogen soluble in neutral permanganate, after the method of the Association of Official Agricultural Chem-

<sup>1</sup> The materials were prepared by research chemists of this company.

ists (1, p. 12), of the materials are given in Table I. Since the availability of the insoluble nitrogen was being studied, all samples, including cottonseed meal, tankage, mixed fertilizer, and UAL-37 bases, were washed to remove the soluble nitrogen.

TABLE I  
WATER-INSOLUBLE NITROGEN CONTENT AND NEUTRAL PERMANGANATE  
NUMBER OF MATERIALS

Materials	Nitrogen %	Neutral permanganate number
Cottonseed meal	6.58	94
Process tankage, composite of seven commercials	7.92	89
Garbage tankage	2.85	78
UAL-37 base, superphosphate 30 lbs. $\text{NH}_3$ , 3 days, $110^\circ \text{F}$ .	0.94	96
" " " 20 " " 14 " $110^\circ$ "	0.94	80
" " " 30 " " 14 " $110^\circ$ "	1.13	94
" " " 40 " " 14 " $110^\circ$ "	1.16	96
" " " 30 " " 14 " $70^\circ$ "	0.56	92
" " " 30 " " 14 " $90^\circ$ "	0.68	92
" " " 30 " " 14 " $130^\circ$ "	1.22	87
" " " 30 " " 14 " $160^\circ$ "	1.37	53
" " " 30 " " 7 " $110^\circ$ "	1.10	95
" " " 30 " " 28 " $110^\circ$ "	1.26	94
" " " 30 " " 56 " $110^\circ$ "	1.15	—
" " " 30 " anhydrous $\text{NH}_3$ , and urea-aqueous formaldehyde	1.25	94
UF polymer prepared from aqueous solution pH 5	28.60	89
Unneutralized super-urea-aqueous formaldehyde	1.75	23
4-12-4, N as process tankage	0.94	84
4-12-4, 66% N as UAL-37 base and 34% as 29 lbs. process tankage and 100 lbs. cocoa shell meal	1.03	85
Resin scrap	19.10	97
Ground urea-formaldehyde molded resin	21.00	74

The resin scrap was a composite of several samples of urea-formaldehyde resin waste obtained from the fertilizer industry. The sample contained some molded resin scrap and a rather large proportion of molding powder. The molded resin was secured by grinding a portion of molded plastic to pass a 100-mesh screen. The urea-formaldehyde polymer was obtained by precipitating from an aqueous solution having a pH of 5.0.

The methods made use of in measuring the availability of nitrogen consisted of nitrification and vegetative experiments. In the former, 34 milligrams of nitrogen and a basic treatment consisting of superphosphate, potassium chloride, and sufficient dolomite to bring the soil to pH 6.0 were mixed with 200 grams of equal parts of Norfolk fine sandy loam and Sassafras sandy loam soils. Each cultural treatment was replicated two times. The mixtures were brought to optimum water content, placed in Erlenmeyer flasks which were loosely plugged with cotton, and incubated at  $27^\circ \text{C}$ . After 30, 60, and 90 days, one-fourth of the contents of each culture

was dispersed with distilled water and the nitrates determined colorimetrically (2).

The Norfolk soil was obtained through the courtesy of H. D. Morris of the North Carolina Agricultural Experiment Station, the Sassafras through the cooperation of A. W. Blair of the New Jersey Agricultural Experiment Station, and the Gloucester loam was taken locally.

The basic treatment afforded each of the two-gallon jars of Norfolk soil, utilized in the plant growth tests, consisted of 4.0 grams of superphosphate (16 per cent  $P_2O_5$ ), 0.5 gram of potassium chloride, and sufficient dolomite to bring the soil to pH 6.0. The cultures of Gloucester soil received identical treatments except that 5.0 grams of superphosphate instead of 4.0 grams were added to each. The cultures were moistened and ten days later the growth tests were started. Millet (*Echinochloa frumentacea* Link), tomato (*Lycopersicon esculentum* Mill. var. Bonny Best), and corn (*Zea mays* L. var. Funk Bros. hybrid G-169) were utilized as the test crops. Further details as to cultural treatments are given in the text and summary tables. The entire harvest from each of the various replicates was finely ground, thoroughly mixed, samples taken in duplicate, and the nitrogen content determined after the Gunning Official Method (1, p. 8).

The results of statistical examination of the replicates in the first harvest of the various cultures show that the averages of the yield from three replicates must differ by at least 2.2 grams to be considered significantly different.

#### RESULTS ON NORFOLK SOIL

The results of the nitrification and growth experiments on Norfolk fine sandy loam are presented in Figure 1 A and Table II. Section 1 of this table gives the data obtained with several common sources of insoluble nitrogen.

The highest yield was secured with cottonseed meal. The synthetic insoluble nitrogen in the Urea-Ammonia Liquor-37 base produced a higher yield than process tankage. Garbage tankage depressed the yield. The 4-12-4 with the insoluble nitrogen derived from process tankage produced a better yield than the 4-12-4 with the insoluble nitrogen from Urea-Ammonia Liquor-37, cocoa shell meal, and a very small amount of tankage. The insoluble nitrogen in 4-12-4 fertilizer was inferior to that in the Urea-Ammonia Liquor-37 base.

The nitrogen recovered in the millet tops is given in Table II, column 3. These data rank the materials in the same order as the yield data. The garbage tankage slightly reduced nitrogen recovery and the insoluble nitrogen of the 4-12-4 made with Urea-Ammonia Liquor-37 and cocoa shell meal gave a lower recovery than the Urea-Ammonia Liquor-37 base or the other 4-12-4.

The nitrification data indicate that cottonseed meal and Urea-Ammonia Liquor-37 are about equal in nitrate production. Both are superior to



FIGURE 1. Availability to millet of insoluble nitrogen from different sources. A. With Norfolk fine sandy loam, reading left to right: 1. No nitrogen. 2. Cottonseed meal. 3. Process tankage. 4. Garbage tankage. 5. UAL-37 base. B. With Gloucester loam, reading left to right: 1. No nitrogen. 2. Cottonseed meal. 3. Process tankage. 4. UAL-37 base. 5. 4-12-4, 66 per cent nitrogen as UAL-37 base.



TABLE II

AVAILABILITY OF WATER-INSOLUBLE NITROGEN MEASURED BY PLANT GROWTH AND  
NITRATE FORMATION. GROWTH INDICATOR, MILLET. NORFOLK SOIL.  
0.3 G. OF NITROGEN PER CONTAINER

Cultural treatment	Yield dry wt. in g., av.	N re- moved, mg. per culture, av.	Nitrate nitrogen, parts per million of soil, av.		
			30 days	60 days	90 days
Section 1. Nitrogen from different sources					
Control	4.8	48	10.6	14.6	14.7
Cottonseed meal	17.8	223	49.3	60.5	61.6
Process tankage	10.3	111	31.4	34.3	36.8
Garbage tankage	3.1	42	5.1	14.7	12.1
UAL-37 base	12.7	159	43.7	64.5	79.5
4-12-4, 66% N as UAL-37 base	9.1	104	26.7	43.6	37.8
4-12-4, process tankage	12.1	140	34.9	46.6	34.9
Section 2. Different forms of synthetic insoluble nitrogen					
Ground molded resin	5.1	57	12.1	17.6	16.3
Resin scrap	13.1	151	24.6	44.6	52.4
UAL-37 base, 30 lbs. NH <sub>3</sub>	12.7	159	43.7	64.5	79.5
Superphosphate, 30 lbs. anhy. NH <sub>3</sub> , urea-aqueous formaldehyde	13.5	147	27.8	49.9	57.8
UF polymer from solution, pH 5.0	5.5	65	17.6	35.1	27.6
Section 3. Influence of ammoniation					
Unneutralized super-urea-aqueous formaldehyde	7.8	83	18.5	35.9	27.1
UAL-37 base, 20 lbs. NH <sub>3</sub>	14.4	166	45.6	53.8	52.5
“ “ 30 “ “	13.2	146	32.8	67.8	64.5
“ “ 40 “ “	12.2	156	27.8	68.0	59.4
Section 4. Influence of storage temperature					
UAL-37 base, 2 weeks, 70° F.	12.8	170	32.3	41.1	44.5
“ “ “ “ 90° F.	13.7	194	31.1	59.0	50.8
“ “ “ “ 110° F.	13.2	146	32.8	67.8	64.5
“ “ “ “ 130° F.	13.7	175	38.4	67.4	57.2
“ “ “ “ 160° F.	12.0	140	32.3	56.1	42.8
Section 5. Influence of duration of storage at 110° F.					
UAL-37 base, 3 days	12.7	159	43.7	64.5	79.5
“ “ 7 “	15.5	216	36.5	72.8	63.5
“ “ 14 “	13.2	146	32.8	67.8	64.5
“ “ 28 “	12.7	173	40.8	47.2	50.5
“ “ 56 “	13.5	181	51.2	72.1	60.5

process tankage. Garbage tankage slightly depressed nitrate formation. Distinctly higher nitrate production was secured with the UAL-37 base than with the 4-12-4 made with UAL-37 and cocoa shell meal. Like the growth experiments, this indicates that cocoa shell meal reduces the effi-

ciency of the insoluble nitrogen. A similar result was obtained in subsequent experiments.

It is believed that the observed depressing effect of garbage tankage and cocoa shell meal on nitrification and plant growth may be due to the wide carbon-nitrogen ratio of these materials. It is generally recognized that in organic materials a narrow carbon-nitrogen ratio favors nitrification whereas materials with a wide carbon-nitrogen ratio may retard nitrification and growth. The insoluble residue of the garbage tankage and cocoa shell meal used in these experiments undoubtedly has a rather wide carbon-nitrogen ratio. The importance of this factor in the availability of organic fertilizer materials merits further investigation.

*Forms of synthetic insoluble nitrogen.* The data of Section 2, Table II, show that different forms of synthetic insoluble nitrogen differ widely in their availability as indicated by crop growth and nitrification. The 100-mesh molded resin is only slightly available. Resin scrap, however, nitrified readily and produced good growth. The high availability of this resin scrap is believed to be due to a high proportion of molding powder in the sample. The insoluble nitrogen in the Urea-Ammonia Liquor-37 base and that produced by treating superphosphate with anhydrous ammonia, urea, and aqueous formaldehyde were similar in availability. It should be noticed, however, that the urea-formaldehyde polymer prepared by precipitation from aqueous solution at pH 5.0 was decidedly less available than the Urea-Ammonia Liquor-37 base. This may indicate that precipitation in the presence of the superphosphate increases the availability of the insoluble compounds formed from the urea-formaldehyde reaction.

*Influence of ammoniation.* In the third section of Table II, there is a comparison of synthetic insoluble nitrogen formed in unneutralized superphosphate and in three Urea-Ammonia Liquor-37 bases. Both yield and nitrification data indicate the insoluble nitrogen formed without neutralization of the superphosphate has a low availability. This is believed to be due to the low pH at which it is formed. The rate of ammoniation within the limits of 20 to 40 pounds of ammonia per 1000 pounds superphosphate did not materially influence the availability of the insoluble nitrogen in the Urea-Ammonia Liquor-37 bases. It may be noted, however, that the higher rates of ammoniation gave the higher activity numbers by the neutral permanganate method.

*Influence of storage conditions.* The influence of the temperature and time of storage of Urea-Ammonia Liquor-37 bases on the availability of the synthetic insoluble nitrogen is indicated by the data of Sections 4 and 5 of Table II. Storage temperatures varying from 70° F. to 160° F. for a two-week period did not significantly influence the availability of the nitrogen as indicated by yields or nitrification data, nor did availability change during storage up to 56 days at 110° F. Such data indicate the synthetic in-

soluble nitrogen derived from Urea-Ammonia Liquor-37 is stable under most conditions of storage.

*Availability and activity.* A comparison of the data presented in Table II with the activity of the insoluble nitrogen as determined by the neutral permanganate method, Table I, shows a very poor correlation. Garbage tankage and ground molded resin have permanganate numbers of 78 and 74 respectively, but both are quite unavailable. Likewise, the urea-formaldehyde polymer with an activity of 89 is relatively unavailable. On the other hand, the Urea-Ammonia Liquor-37 base stored at 160° F. had an activity number of 53 but produced good growth and nitrified readily.

#### RESULTS ON GLOUCESTER LOAM

*First crop.* The vegetative experiments on Gloucester loam were similar to those on the Norfolk fine sandy loam except the number of treatments was reduced. Corn, tomatoes, and millet were used as test crops. The corn and tomatoes received 0.50 gram insoluble nitrogen and the millet cultures 0.30 gram nitrogen. The average dry weights in grams from each cultural treatment and the average nitrogen removal by the above-ground portions of the corn, tomato, and millet plants are presented in Table III and Figure 1 B.

TABLE III

AVAILABILITY OF WATER-INSOLUBLE NITROGEN, 0.5 GRAM N ADDED TO CORN AND TOMATO AND 0.3 GRAM N TO MILLET CULTURES. GLOUCESTER LOAM. YIELD DRY WEIGHT IN GRAMS. NITROGEN REMOVED IN MILLIGRAMS

Cultural treatment	Corn		Tomato		Millet	
	Average yield, g.	Mg. N removed, av.	Average yield, g.	Mg. N removed, av.	Average yield, g.	Mg. N removed, av.
1 No nitrogen	4.8	42	5.5	63	5.0	57
2 Cottonseed meal	20.5	266	16.0	253	12.8	194
3 Process tankage	13.2	132	8.0	141	8.5	110
4 UAL-37 base	21.0	248	15.5	262	12.5	183
5 4-12-4, 66% N as UAL-37 base	13.9	124	11.2	162	7.8	102
6 4-12-4, all N as process tankage	18.9	172	15.9	222	9.6	135
7 Unneutralized superphosphate-urea-aqueous formaldehyde	9.5	81	6.1	41	6.1	118

The data of Table III indicate cottonseed meal and the synthetic insoluble nitrogen of Urea-Ammonia Liquor-37 base are of equal efficiency. Both produced excellent and substantially equivalent increases in the yields of all three crops. Nitrogen removal from the cottonseed meal and UAL-37 base cultures averaged 238 and 231 milligrams respectively, as compared to 54 milligrams in the control or no nitrogen culture. Process tankage produced about 50 per cent as large an increase in growth as did cottonseed

meal and the UAL-37 base. The average nitrogen removal by the tankage was 128 milligrams nitrogen.

A comparison of cultures No. 5 and No. 6 shows that the insoluble nitrogen from 4-12-4 made with process tankage produced better yields than the insoluble nitrogen of the 4-12-4 made with Urea-Ammonia Liquor-37 and cocoa shell meal. Both 4-12-4 cultures produced smaller yields than the UAL-37 base. It is apparent, therefore, that the insoluble nitrogen in cocoa shell meal is of little or no value in promoting crop growth. As previously indicated, it may tend to depress yields because of its wide carbon-nitrogen ratio.

Culture No. 7, synthetic insoluble nitrogen formed in unneutralized superphosphate, produced very poor yields of all three crops. This again shows the importance of neutralizing superphosphate prior to the formation of the insoluble nitrogen compounds. This, of course, is accomplished by the use of Urea-Ammonia Liquor-37 but might also be accomplished with other alkaline materials.

*Second crop.* After the harvest of plants grown in the various cultures, the roots were removed, the soil, to which 2 grams superphosphate were added, mixed, returned to the jars, and millet seed sown. The carry-over or the residual effect of the nitrogen was measured by the growth of the millet crop. The results derived from this series are presented in Table IV.

TABLE IV  
RESIDUAL EFFECT OF INSOLUBLE NITROGEN. CROP INDICATOR, MILLET.  
GLOUCESTER LOAM. YIELD IN GRAMS

Culture No.	Cultural treatment	Following tomatoes		Follow- ing corn, av.	Follow- ing millet, av.
		Average weight	Mg. N removed, av.		
1	No nitrogen	0.73	7.04	0.55	0.67
2	Cottonseed meal	3.22	27.83	1.33	1.25
3	Process tankage	2.1	17.81	1.07	0.67
4	UAL-37 base	6.11	49.06	3.33	2.95
5	4-12-4, 66% N as UAL-37 base	2.61	21.85	1.76	1.35
6	4-12-4, all N as process tankage	2.95	26.0	1.39	1.07
7	Unneutralized superphosphate-urea-aqueous formaldehyde	1.97	16.83	0.80	1.19

The best growth was secured following tomatoes. In that series, as well as following corn and millet, the residual value of the insoluble nitrogen from Urea-Ammonia Liquor-37 base was more than twice that of cottonseed meal. The residual value of process tankage was lower than that of cottonseed meal. The low availability of the synthetic insoluble nitrogen formed in unneutralized superphosphate, culture No. 7, is shown by its slight residual value. Despite the fact that it made a very poor first crop, the second crop was poorer than any of the other cultures except the control.



## SUMMARY AND CONCLUSIONS

Millet grown in Norfolk fine sandy loam treated with insoluble synthetic nitrogen formed from Urea-Ammonia Liquor-37 under proper conditions gave slightly lower yields than it did where the water-insoluble nitrogen was added in the form of cottonseed meal, but a greater harvest than accrued from the use of process tankage. The synthetic insoluble nitrogen, however, was as available to millet, corn, and tomato plants when added to Gloucester loam as was cottonseed meal. The nitrogen in ground molded resin and garbage tankage, when added to the Norfolk soil, was not available to millet. On the other hand, the nitrogen in resin scrap was available to it.

Storage of UAL-37 base at 110° F. over a period of eight weeks did not alter its availability. Moreover, storage for two weeks under temperatures ranging from 70° F. to 160° F. did not affect it in this respect.

The residual or carry-over effect of insoluble nitrogen in UAL-37 base was greater than it was in cottonseed meal as evidenced by the yield of the second crop of millet grown in Gloucester loam.

A comparison of the rate of nitrification of insoluble nitrogen from different sources revealed UAL-37 base and cottonseed meal to be the most active, that in process tankage and the mixed fertilizers less so, and garbage tankage retarded nitrate formation in the soil.

The importance of ammoniation of the superphosphate and the formation of the insoluble nitrogen at a proper reaction were demonstrated by both growth and nitrification studies. Ground molded resin proved to be of little value as a source of nitrates. The temperature and time of storage did not affect the rate of nitrification of UAL-37 base.

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STUDIES ON THE DEVELOPING COTTON FIBER. III.  
THE SPECIFIC VISCOSITY OF CUPRAMMONIUM  
HYDROXIDE DISPERSIONS OF DEVELOPING  
COTTON FIBERS AND AN ESTIMATION OF  
THE CALCIUM PECTATE NUMBERS<sup>1,2</sup>

JACK COMPTON

The presence of cellulose in the cotton fiber at all stages of development has been shown by optical methods in both ordinary and polarized light and by microchemical tests (13). X-ray analysis of the developing cotton fiber shows the presence of crystalline cellulose at about 30 to 35 days in the untreated fibers, between 15 to 20 days in fibers extracted with organic solvents, and as early as 5 days in carefully purified fibers (24). As discussed by Farr (10), cotton fibers are formed by the outward extension of single cells from the epidermis of the seed. This extension begins between the day of flowering and the eleventh or twelfth day, and may even be formed on some parts of the seed surface after twenty days. According to Armstrong and Bennett (1) and others (3, 17), cotton fiber elongation continues until about the twenty-fifth to thirtieth day with little or no change in cell diameter. During rapid fiber elongation the fiber wall is extremely thin and delicate, but during the next 20 to 25 days the cell wall thickens by the deposition of successive layers, or lamellae, of cellulose and other constituents of the cytoplasm (10, 12). It is during this period also that a preferred orientation of the cellulose develops with reference to the fiber axis (25), and the spiral arrangement of the fibrils becomes evident. Fibril formation from cellulose particles ( $1.1 \times 1.5 \mu$ ) existing in the fiber cytoplasm has been reported by Farr and Eckerson (13) who have also shown that the fibrils may be disintegrated into cellulose particles of approximately the same dimensions (14). Bailey and Brown (2) have recently confirmed the observation that fibrillae are about  $1 \mu$  in diameter and composed of uniform microscopic units.

On the fifty-fifth to sixtieth day after flowering, when the cotton boll normally opens, the age of the individual cotton fibers attached to a single seed may extend over a period of from perhaps 35 to 60 days. Although the percentage of the thin-walled "immature" cotton fibers which may thereby result is usually relatively low, nevertheless many of the physical properties of raw, and perhaps of purified, cotton are decidedly affected by their presence (22). As pointed out by Pearson (21), from a practical point of

<sup>1</sup> Presented before the Division of Cellulose Chemistry at the 99th Meeting of the American Chemical Society, Cincinnati, Ohio, April 10, 1940.

<sup>2</sup> Cellulose Department, Chemical Foundation, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York.

view, such fibers are a potential source of neps, the removal of which increases the cost of finished textile products.

Since many of the physical properties of cotton fibers may be correlated with the cuprammonium viscosity (9, p. 48-75), the present investigation was undertaken to study the cuprammonium dispersibility of cotton fibers at different stages of development and to determine the specific viscosity of the resulting dispersions. Also, since pectic substances apparently play an important rôle in fiber development (20, p. 74-101; 23) and affect both the physical and chemical properties of cotton fibers at maturity (11), the calcium pectate numbers of the developing cotton fibers have been determined.

## EXPERIMENTAL

### DISPERSIBILITY OF THE DEVELOPING COTTON FIBER IN CUPRAMMONIUM HYDROXIDE SOLUTION AND THE SPECIFIC VISCOSITY OF THE RESULTING DISPERSIONS

The cotton fibers used in this investigation were obtained from fresh cotton bolls grown on cotton plants, Super Seven variety (*Gossypium hirsutum* L.), under normal field conditions at the South Carolina Experiment Station, Clemson College, South Carolina, during the summers of 1938 and 1939. The method of selecting the bolls has been described in a previous publication (8).

#### *Purification Treatments*

*A. Extraction with water and alcohol-benzene (1:1).* After removal of the cotton fibers from the seed the fresh fibrous material was placed into ice-water, thoroughly washed with water and allowed to air-dry at room temperature. The air-dried material was placed in a Soxhlet apparatus and extracted with alcohol-benzene (1:1) for 24 hours. The crude cotton fiber was then air-dried at room temperature in a well ventilated cabinet. Upon standing for several days the weight of the mass became constant. The extent to which the crude fibers, 1938 crop, dispersed in cuprammonium hydroxide solution and the specific viscosities of the resulting dispersions are given in column 2, Table I, and column 6, Table II, respectively.

The effect of first extracting the dry crude developing cotton fiber, 1939 crop, with alcohol-benzene (1:1) and then with water upon the dispersibility in cuprammonium hydroxide solution and the specific viscosities of the resulting dispersions is given in Table III.

*B. Extraction with 0.5 per cent ammonium oxalate solution at 80° C.* Ten grams of the crude cotton fibers, previously extracted with water and alcohol-benzene, (A) above, were cut across with shears into approximately 2 mm. lengths, placed in a 800 cc. Pyrex test tube and 300 cc. of 0.5 per cent ammonium oxalate solution heated to 90° C. added. After thorough stirring



TABLE I

EFFECT OF VARIOUS PURIFICATION TREATMENTS OF DEVELOPING COTTON FIBERS (1938 CROP) UPON THE DISPERSIBILITY IN CUPRAMMONIUM HYDROXIDE SOLUTION AND NATURE OF THE RESULTING DISPERSIONS

Days after flow- ering	Effect of various purification treatments upon per cent dry crude cotton fiber:											
	(a) Dispersible in cuprammonium solution				(b) Cellulose coagulated from acidified cuprammonium dispersions				(c) Components remaining in solution after acidification of cuprammonium dispersions			
	A*	B	C	D	A	B	C	D	A	B	C	D
10	57.2	58.7	—	81.4	12.8	35.1	—	51.5	44.3	23.7	—	28.9
15	75.1	83.4	93.8	96.6	64.7	72.5	93.8	74.2	10.4	10.9	0.0	22.4
20	95.2	75.3	93.5	96.0	39.2	64.6	93.5	81.9	25.9	10.7	0.0	14.1
25	90.9	85.1	97.2	99.0	82.5	78.6	97.2	93.0	8.4	6.5	0.0	6.0
30	88.1	93.8	97.2	100.0	80.9	85.6	97.2	97.0	7.2	8.1	0.0	3.0
35	86.7	85.5	92.8	100.0	81.1	81.1	92.8	98.4	5.6	4.4	0.0	1.6
40	90.8	99.4	95.6	100.0	81.9	95.7	95.6	100.0	8.9	3.7	0.0	0.0
45	94.8	96.8	98.4	100.0	87.1	96.8	98.4	100.0	7.7	0.0	0.0	0.0
50	94.3	96.6	96.4	100.0	89.2	96.6	96.4	100.0	5.1	0.0	0.0	0.6
55	98.1	97.8	100.0	100.0	97.5	97.8	100.0	—	0.6	0.0	0.0	—

\* A, extraction with water and alcohol-benzene (1:1); B, similar to A plus extraction with 0.5 per cent ammonium oxalate solution at 80° C.; C, similar to B plus extraction with 1 per cent sodium hydroxide solution for 4 hours at 80° C. in nitrogen atmosphere; D, similar to A plus direct extraction with 1 per cent sodium hydroxide solution for 4 hours at 80° C. in a nitrogen atmosphere.

TABLE II

EFFECT OF VARIOUS PURIFICATION TREATMENTS OF DEVELOPING COTTON FIBERS (1938 CROP) UPON THE SPECIFIC VISCOSITY AND CALCULATED DEGREE OF POLYMERIZATION OF THE CUPRAMMONIUM HYDROXIDE DISPERSIONS

Days after flow- ering	Concentration of cellulose [Table I (b)], C, g./l.				$\eta_{sp}/C$				Calculated degree of polymerization (D.P.) $K_m = 5 \times 10^{-4}$			
	A*	B	C	D	A	B	C	D	A	B	C	D
10	0.292	0.472	—	0.132	0.983	0.793	—	1.048	1966	1586	—	2097
15	0.150	0.193	0.234	0.186	1.153	1.004	1.105	1.439	2306	2008	2210	2879
20	0.091	0.172	0.230	0.205	1.153	1.046	1.081	1.410	2306	2092	2162	2820
25	0.195	0.206	0.243	0.240	1.682	1.387	1.273	1.769	3364	2774	2545	3538
30	0.191	0.224	0.242	0.241	1.659	1.465	1.310	1.785	3318	2930	2620	3569
35	0.192	0.211	0.231	0.246	1.624	1.525	1.440	1.788	3248	3050	2879	3577
40	0.194	0.248	0.239	0.250	1.337	1.391	1.325	1.691	2670	2782	2650	3383
45	0.207	0.248	0.246	0.250	1.459	1.258	1.336	1.713	2918	2516	2672	3426
50	0.210	0.240	0.241	0.250	1.415	1.518	1.430	1.807	2930	3035	2860	3613
55	0.231	0.250	0.250	0.250	1.413	1.210	1.477	—	2826	2420	2953	—

\* See footnote Table I.

the mixture was heated at 80° C. in a closed system<sup>3</sup> for 24 hours, with in-

<sup>3</sup> Unless a closed system is used, the ammonium oxalate decomposes to a slight extent at this temperature with the liberation and loss of ammonia. The solution will accordingly become sufficiently acid to lower appreciably the cuprammonium viscosity of the fiber system.

termittent agitation. At the end of this time the fibers were separated by filtration and thoroughly washed on an open Büchner funnel with hot water by the application of suction. Finally the moist fibers were again placed in the test tube and extracted with 0.5 per cent ammonium oxalate solution as described above. This process was thrice repeated (16), the combined extracts and washings being saved in each case for determining the calcium pectate number of the cotton fibers as described below.

In the case of the 10-, 15-, and 20-day cotton fibers, where only 1, 2, and 5 grams of material were available, respectively, the procedure was modi-

TABLE III

EFFECT OF VARIOUS PURIFICATION TREATMENTS OF DEVELOPING COTTON FIBERS (1939 CROP) UPON THE DISPERSIBILITY IN CUPRAMMONIUM HYDROXIDE SOLUTION AND THE SPECIFIC VISCOSITY AND CALCULATED DEGREE OF POLYMERIZATION OF THE DISPERSED FIBER PHASE

Days after flowering	Cotton fiber dispersed in cuprammonium solution, %				$\eta_{sp}/C$				Calculated degree of polymerization (D.P.) $K_m = 5 \times 10^{-4}$			
	A*	B	C	D	A	B	C	D	A	B	C	D
10	12.6	17.9	—	60.9	0.3454	0.3714	—	0.7641	690	743	—	1528
15	8.2	14.5	3.0	70.4	0.6032	0.6308	0.5614	1.0460	1206	1261	1123	2092
20	56.8	63.7	58.6	100.0	1.3671	1.3060	1.0240	1.1896	2734	2612	2048	2378
25	75.0	83.3	86.4	100.0	1.4349	1.3952	1.2815	1.3192	2869	2790	2563	2638
30	78.2	90.7	90.4	100.0	1.4390	1.3967	1.4385	1.4728	2878	2793	2877	2945
40	86.6	90.2	88.8	100.0	1.4097	1.5555	1.4536	1.4188	2819	3111	2907	2837
50	89.7	93.0	92.1	100.0	1.4686	1.3832	1.2124	1.1756	2937	2766	2425	2351
55	88.0	92.2	98.6	100.0	1.4368	1.6946	1.6102	1.4135	2873	3389	3220	2827

\* A, Raw untreated cotton fibers; B, cotton fibers extracted with alcohol-benzene (1:1); C, similar to B plus extraction with water; D, similar to B plus extraction with 1% sodium hydroxide solution for 4 hours at 80° C. in a nitrogen atmosphere.

fied accordingly. It was also necessary to use sintered glass filters No. 2, in place of the open Büchner funnel to retain the fibers of this age during the filtering and washing processes.

After the final oxalate extraction, the fibrous residues in each case were washed with hot water until all the soluble salts were removed, thoroughly washed with 95 per cent ethyl alcohol and air-dried at room temperature. The effect of this treatment upon the dispersibility of the cotton fibers in cuprammonium hydroxide solution and the specific viscosities of the dispersions is given in column 3, Table I, and column 7, Table II, respectively.

*C. Extraction with 1 per cent sodium hydroxide solution at 80° C. for 4 hours in a nitrogen atmosphere.* One gram of the crude cotton fibers, after having received treatments (A) and (B) above, were extracted with 200 cc. of a 1 per cent sodium hydroxide solution at 80° C. for 4 hours in a nitrogen atmosphere. The alkali was pre-boiled and without cooling was poured on the cotton fibers and allowed to boil under diminished pressure for several

minutes to expel the entangled air in the fibers. Alternately, nitrogen pressure and vacuum were applied to the closed system to remove the last traces of air. The stoppered pressure bottles were then immersed in a water-bath heated to 80° C. and allowed to remain with occasional shaking for 4 hours. The bottles were then cooled, opened, and the fibers separated by filtering on a Büchner funnel. The fibers were then given a rapid preliminary washing with distilled water and placed in 200 cc. of ice-cold 0.5 per cent sulphuric acid solution. After 10 to 15 minutes' stirring, the fibers were removed by suction filtration and thoroughly washed with cold water. Finally, the fibrous mass was removed to a beaker and allowed to stand overnight in a large volume of water, separated by filtration, washed with 95 per cent ethyl alcohol, and air-dried at room temperature. The results obtained after this treatment are recorded in Tables I and II, column C.

In a similar manner, one gram of cotton fibers at each stage of development, previously extracted with water and alcohol-benzene as described in (A) above, were treated with 1 per cent sodium hydroxide solution at 80° C. for four hours in a nitrogen atmosphere. The data obtained from fibers treated in this manner are given in Tables I, II, and III, column D.

*Method Employed in Dispersing the Cotton Fibers in Cuprammonium Hydroxide Solution and the Specific Viscosity of the Resulting Dispersions*

The cuprammonium hydroxide solution used was prepared according to the method of Clibbens and Geake (4), and contained 15 grams of copper, 240 grams of ammonia, and less than 0.5 gram of nitrous acid per liter. Samples of the cotton fibers, previously cut across with shears into lengths not exceeding 2 mm., were taken of such a weight as to yield 0.5 per cent fiber dispersions (0.5 gram fiber in 100 cc. solution, moisture-free basis). The samples were conveniently weighed on a small watch-glass and quantitatively transferred to the dispersion tubes of known volumes. A few small glass beads of known volume displacement were then added to each tube, the tube half-filled with cuprammonium hydroxide solution, and the fibers stirred gently into the solvent for a few seconds to release entangled air. The tubes were then filled with the dispersing agent to within an inch of the top, the rod being washed at the same time. Finally, the tubes were filled to a point slightly above the calibration marks for the stoppers with cuprammonium solution and the stopper inserted so that the liquid in slight excess, displacing all air, overflowed through the capillary tubes in the stoppers. The rubber tubes attached to the capillary tubes were then folded over and made tight with rubber bands. The tubes were then vigorously shaken to set the glass beads in motion, securely fastened in a box, and placed in an apparatus constructed so as to make four revolutions per minute. The end-over-end motion of the tubes and the resulting motion of

the glass beads produced an agitation in the liquid which was continued until homogeneous dispersions were obtained. A uniform period of 24 hours was taken for the dispersion, since previous experiments had shown this to be the optimum dispersion time for this material. The mixtures were maintained at a temperature of 4° C. during dispersion by placing the entire apparatus in a cold room thermostatically controlled. Usually seven tubes were run at the same time in this manner. This procedure, when carefully followed, insured the maximum dispersion of the cotton fibers, the absence of air, and reproducibility of results.

*Viscosity measurements.* After the dispersion process was completed the tubes were removed from the box and centrifuged for 30 minutes at 2000 r.p.m., cooled to 5° C., and opened in a nitrogen atmosphere. An aliquot of the dispersions was then taken from the mid-portion of the tubes with a calibrated pipette and transferred to 10 cc. volumetric flasks. The exact amount of the initial dispersions to be diluted was determined by the viscosity of the dispersion. In most cases, however, a pipette calibrated to contain 0.5 cc. was employed. The pipette was allowed to drain under nitrogen pressure and was then washed free of the dispersion with cuprammonium solution into the volumetric flask. Finally, cuprammonium solution was added to the mark and the solution thoroughly mixed. The air in the pipette and volumetric flasks had previously been displaced with nitrogen gas and the dispersions were at no time allowed to contact the air. Exactly 5 cc. of the diluted dispersions were then transferred in a nitrogen atmosphere to a calibrated Ostwald viscometer held in a thermostat at 25° C. After the dispersions in a nitrogen atmosphere had attained the temperature of the thermostat, the time of flow of each was noted with a stop watch until checks within 0.1 second were obtained. The exclusion of oxygen during transference makes it possible to obtain checks after 5 to 10 minutes.<sup>1</sup> The time required for diluting the initial dispersions and determining the time of flow in the viscometer was usually 20 to 25 minutes.

Substituting the observed time of flow of each of the dispersions in the equation,  $\eta_{cp} = dt/C$ , the viscosities expressed in centipoise units are obtained. In this equation,  $d$  is the density of the dispersion at 25° C.,  $t$ , observed time of flow in seconds, and  $C$ , a constant characteristic of the viscometer. The value of  $C$  is found from this same equation when the time of flow and density of a liquid of known viscosity ( $\eta_{cp}$ ) is known. With distilled water as a standard the following data were obtained for the viscometer used:  $t$  (secs.), 75.6;  $d^{25^\circ}$ , 0.99707;  $\eta_{cp}^{25^\circ}$ , 0.893, and hence the calculated value of  $C$ , 8460.2. For the cuprammonium solution the following data

<sup>1</sup> If checks cannot be obtained the run is not necessarily lost. By plotting the time of flow against the time of standing in the thermostat and extrapolating to zero, the initial time of flow may be accurately determined if proper precautions are taken.



were obtained:  $t$  (secs.), 111.6;  $d^{26^\circ}$ , 0.920, and hence the calculated viscosity  $\eta_{cp}^{25^\circ}$ , 1.2136.

The relative viscosity ( $\eta_{rel.}$ ) of the cotton fiber dispersions may now be calculated using the equation  $\eta_{rel.} = \frac{\eta_{cp, \text{dispersion}}}{\eta_{cp, \text{cuprammonium solution}}}$ , in which  $\eta_{cp}$ , dispersion is the viscosity of the cotton fiber dispersion in centipoise units and  $\eta_{cp}$ , cuprammonium solution, the viscosity of the cuprammonium solution in centipoise units.

The increase in viscosity of the solvent due to the dispersed fiber phase (26) or specific viscosity ( $\eta_{sp}$ ) is obtained from the relative viscosity ( $\eta_{rel.}$ ) by use of the equation  $\eta_{sp} = \eta_{rel.} - 1$ .

#### *Determination of the Concentration of Cellulose in Cuprammonium Hydroxide Dispersions of Cotton Fibers*

After the dispersion process of the cotton fibers in cuprammonium hydroxide solution had been completed, the undispersed fiber residue was separated from the dispersed fiber phase by centrifuging, as previously described. An aliquot of each dispersion was removed with a pipette calibrated to contain exactly 10 cc., drained into a 200 cc. beaker, and the pipette thoroughly rinsed with water. The dispersion was then diluted to 100 cc. with ice-water and the mixture acidified with cold 25 per cent sulphuric acid, using Congo Red indicator. After 30 minutes in the cold the coagulated cellulose was transferred to a sintered glass filter of known weight, thoroughly washed with water, and dried to constant weight at  $105^\circ$  C. From the weight of the cellulose obtained from the aliquot, the total weight of this dispersed fiber phase can be calculated. This value, divided by the weight of the crude cotton fiber, gives the per cent of this dispersed fiber component. The data for the developing cotton fiber, following the various purification processes previously described, are given in Table I, columns 6 to 10. Typical X-ray diffraction patterns for mercerized cellulose were obtained for the material coagulated from cuprammonium dispersions of 10-day and 30-day cotton fibers (Fig. 1).

Knowing now the concentration of the cellulose in the original dispersions, the concentration in the dilute dispersions used in the viscosity studies may be expressed in grams per liter. These values for each sample are given in Tables II and III.

#### *Determination of $\gamma$ -Cellulose in Cuprammonium Dispersions of Cotton Fibers*

The cotton fiber residues centrifuged from the cuprammonium dispersions were washed twice with 10 cc. portions of cuprammonium solution and twice with water by centrifugation. The residues were then made acid

to Congo Red indicator, washed with water until free of salts, and dried to constant weight at  $105^{\circ}\text{C}$ . The loss in weight of the cotton fiber upon treatment with cuprammonium solution can now be calculated. Upon subtracting the weight of the cellulose precipitated from the dispersions by acidification ( $\alpha$ - and  $\beta$ -celluloses) from the weight of the fiber dispersed by cuprammonium solution, the weight of the fiber remaining in the acidified dispersion mixture was obtained ( $\gamma$ -cellulose). These values expressed as per cent of the dry crude fiber are given in Table I, columns 10 through 13.

*Calculation of the  $\eta_{sp}/C$  Values and the Degree of Polymerization of Cellulose Dispersed in Cuprammonium Hydroxide Solution*

The specific viscosity of the dilute cuprammonium hydroxide dispersions divided by the concentration of the cellulose ( $\alpha$ - and  $\beta$ -celluloses),

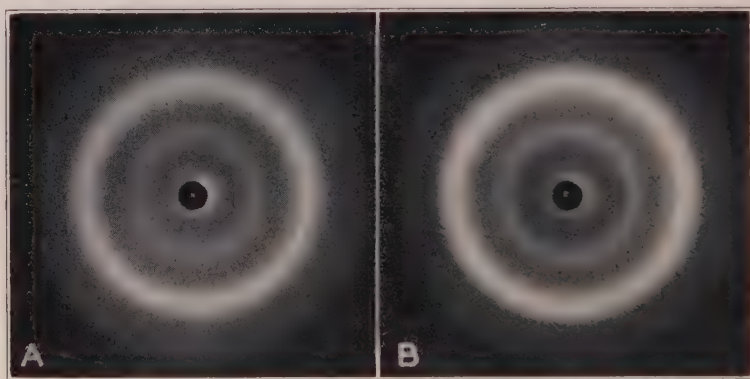


FIGURE 1. X-ray diffraction patterns of cellulose coagulated from cuprammonium hydroxide dispersions of (A) 10-day cotton fibers, and (B) 30-day cotton fibers.

expressed in grams per liter, gives the specific viscosity per unit concentration,  $\eta_{sp}/C$ . These values are recorded in Tables II and III.

The empirical equation of Staudinger (26, p. 475-498),  $\text{D.P.} = \eta_{sp}/C \cdot K_m$ , was employed for calculating the degree of polymerization (D.P.) of the cellulosic component of the developing cotton fibers. In this equation,  $K_m$  is a constant which for cuprammonium dispersions of cellulose has a value  $K_m = 5 \times 10^{-4}$ . The results obtained upon substituting the  $\eta_{sp}/C$  values obtained, as described above, in this equation are given in Tables II and III.

ESTIMATION OF THE CALCIUM PECTATE NUMBERS  
OF THE DEVELOPING COTTON FIBER

The calcium pectate numbers of the developing cotton fibers (1939 crop) were determined by an adaptation of the procedure described by Nanji and Norman (19).

The method used for extracting the cotton fibers with 0.5 per cent am-

monium oxalate solution has been fully described under Purification Treatments (B), above. The combined ammonium oxalate extracts and washings thus obtained, which were approximately 1500 cc. in each case, were concentrated under diminished pressure at 40° C. to about 200 cc., filtered through a thin layer of Celite, and the combined clear filtrate and filter washings dialyzed for 15 to 18 hours against distilled water. The solution was then removed from the dialyzer, concentrated under diminished pressure at 40° C. to 100 cc., and diluted with 350 cc. of cold 95 per cent ethyl alcohol acidified with 5 or 6 drops of concentrated hydrochloric acid. After standing overnight at 5° C. the precipitate was collected on a fluted filter paper and washed with acid alcohol until free of oxalate. The paper and precipitate were placed in a beaker and the solid dissolved by boiling with 50 cc. of very dilute ammonium hydroxide. The solution was filtered and the paper boiled with water. Finally, water was again added to the paper which was triturated with a glass rod, filtered off, and washed with hot water and hot dilute ammonium hydroxide solution. The total liquid usually over 100 cc. was treated after cooling with 100 cc. of 0.4 per cent sodium hydroxide solution and allowed to stand overnight at 5° C. To the solution there was then added 50 cc. of 1 N acetic acid, together with 50 cc. of 11.1 per cent (1 M) calcium chloride solution and the mixture boiled for five minutes. The calcium pectate was filtered off as hot as possible on No. 3 sintered glass filters of known weight. The gel was washed with water till free from chloride and the precipitate dried at 105° C. and weighed. The calcium pectate numbers were then calculated to correspond to the weight of calcium pectate that would be obtained from 100 grams of the cotton fibers at each stage of development. The results obtained for the developing crude cotton fiber are given in Table IV. The results similarly obtained for the crude cotton fibers (1938 crop) after extraction with water and alcohol-benzene (1:1) are also given in Table IV, column 8.

#### DETERMINATION OF HYGROSCOPIC MOISTURE OF DEVELOPING COTTON FIBER AFTER VARIOUS PURIFICATION TREATMENTS

The hygroscopic moisture content of the developing cotton fiber after the various purification treatments previously described is summarized in Table V. The determinations were made in the usual manner (9, p. 18-19) by heating a portion of the various fiber samples at 105° C. for 24 hours.

#### RESULTS AND DISCUSSION

The purification of cotton fibers as ordinarily carried out in commercial processes causes, in varying degrees, modification of the fiber components and disintegration of the fiber structure. In the present investigation, purification procedures have been selected which have previously been found to cause the least fiber disintegration. Since the viscosity of cellulosic materials dispersed in cuprammonium hydroxide solution can in

many cases be correlated with fiber disintegration (9, p. 140, 163, 167), this criterion has been selected in determining the desirability of the methods.

TABLE IV  
CALCIUM PECTATE NUMBERS OF THE DEVELOPING COTTON FIBER (FIELD COTTON,  
1939 CROP)

Days after flowering	Wt. of dry crude cotton fiber per boll, g.	Water soluble constituents of cotton fiber, %	Wt. of dry crude cotton fiber after extraction with water per boll, g.	Wt. of calcium pectate obtained from dry crude cotton fiber per boll, mg.	Calcium pectate number of dry crude cotton fiber	Calcium pectate number of dry crude cotton fiber after extraction with water	
						1939 crop	1938 crop
10	0.097	76.3	0.023	5.7	6.5	24.9	21.6
15	0.179	76.0	0.043	6.9	4.2	16.0	10.4
20	0.264	35.0	0.172	8.5	3.4	4.9	8.4
25	0.425	29.2	0.301	7.6	1.9	2.5	2.3
30	1.018	11.8	0.898	10.6	1.1	1.2	1.7
35	—	—	—	—	—	—	1.8
40	1.581	10.2	1.420	11.3	0.8	0.8	0.9
45	—	—	—	—	—	—	0.8
50	1.219	6.6	1.140	9.6	0.8	0.8	1.1
55	—	—	—	—	—	—	1.1

The procedures employed for the purification of the developing cotton fibers have thus consisted of: (A) extraction with water and alcohol-benzene (1:1), to remove the water-soluble fiber constituents and the fiber fats and waxes; (B) similar to treatment (A), with further extraction with 0.5

TABLE V  
HYGROSCOPIC MOISTURE CONTENT OF THE DEVELOPING COTTON FIBER AFTER  
VARIOUS PURIFICATION TREATMENTS

Days after flowering	Crude cotton fiber, %	Crude cotton fiber after water extraction, %	Crude cotton fiber after water and 0.5% ammonium oxalate extraction, %	Crude cotton fiber after water, 0.5% ammonium oxalate and 1% NaOH extraction, %	Crude cotton fiber after water, alcohol-benzene (1:1) and 1% NaOH extraction, %
10	20.8	10.2	7.1	—	5.0
15	17.9	7.4	0.4	4.5	4.4
20	7.3	7.0	6.1	4.3	4.6
25	7.0	5.4	4.5	3.6	3.4
30	4.5	5.8	4.2	4.0	3.5
40	4.3	5.1	4.9	3.6	3.6
50	4.5	5.3	5.4	3.5	3.6

per cent ammonium oxalate to remove free pectin, pectin combined with metallic ions and pectic acid, free and combined; (C) similar to (B) with additional treatment with 1 per cent sodium hydroxide solution for four hours at 80° C. in a nitrogen atmosphere; and (D) similar to (A) with direct extraction with 1 per cent sodium hydroxide solution for four hours at



80° C. in a nitrogen atmosphere. Although it is known that the prolonged extraction of cotton fibers with sodium hydroxide solution causes fiber disintegration and lowers the cuprammonium viscosity (15), control experiments showed that no appreciable change occurred under the conditions employed, (C) and (D). The treatment with alkali removes fats and waxes, pectic materials, nitrogenous substances, hemicelluloses, and other fiber constituents including inorganic salts.

The dispersibility of cotton fibers in cuprammonium hydroxide solution as a function of age and purification treatments is given in Tables I and III. It will be noted that the per cent of the fiber which disperses in this reagent increases with age, the maximum being attained with cotton fibers 25 to 30 days old and remaining practically constant thereafter. With further purification the fibers disperse to a greater extent but the maximum in each case is attained at approximately the same age and maintained thereafter to maturity. It is perhaps significant that this minimum age corresponds with the period when fiber elongation practically ceases and, "secondary wall" formation, principally cellulose deposition, is most rapid.

In columns 6 to 10, Table I, the percentages of the dispersed cotton fibers, which correspond in behavior to  $\alpha$ - and  $\beta$ -celluloses as a function of age and purification treatments are given, and in columns 10 through 13, Table I, the percentage of the dispersed fiber corresponding to  $\gamma$ -cellulose is given. It will be observed that with the 10-day cotton fiber only 12.8 per cent of the 57.2 per cent of the fiber which dispersed corresponds to ordinary cellulose. The X-ray pattern of this material was typical of that given by mercerized cellulose, Figure 1. The remainder of the dispersed fiber, 44.3 per cent, did not coagulate when the dispersion was acidified, and thus fell in the category of  $\gamma$ -cellulose or hemicelluloses.

When the 10-day cotton fiber, 1938 crop, column 2, Table I, was further extracted with ammonium oxalate solution, the dispersibility of the fiber was only slightly increased, column 3, Table I, but the cellulose content of the dispersion increased nearly three-fold with an approximately 50 per cent decrease in the  $\gamma$ -cellulose content. This would seem to indicate that only a fraction of the total pectic material present in the water and alcohol-benzene extracted fiber was dispersed by the cuprammonium and that the fraction remaining in the fiber prevented the complete dispersion of the cellulose. This was equally true but to a lesser degree with the fibers of other ages.

Upon treating the same fibers, i.e., after treatments (A) and (B), Table I, with sodium hydroxide solution in the absence of air, the dispersibility of the fiber and per cent of cellulose in the dispersions were still further increased, columns 4 and 8, Table I, with the complete removal of the  $\gamma$ -cellulose component, column 12, Table I. If, however, the cotton fibers, after extraction with water and alcohol-benzene, were extracted directly

with sodium hydroxide solution in the absence of air the maximum dispersibility of the fibers was attained, column 5, Tables I and III. However, of the 96.6 per cent of the 15-day fiber, 1938 crop, which dispersed, only 74.2 per cent was cellulose, while 22.4 per cent appeared to be hemicelluloses. The treatment of the cotton fibers directly with sodium hydroxide solution for four hours at 80° C. in an inert atmosphere<sup>5</sup> would thus appear to be less efficient as a purifying agent than when the fibers are first treated with ammonium oxalate solution and then extracted with alkali. Obviously, however, if the extraction time with alkali was extended the hemicelluloses would be completely removed. A certain amount of fiber disintegration would thus result but perhaps no more than with the ammonium oxalate treatment.

Upon comparing column 2, Table I, with column 4, Table III, it would appear that extraction of developing cotton fibers with water and then with alcohol-benzene gives better dispersion properties than when this process is reversed. It should be pointed out, however, that the extent to which cellulosic fiber materials disperse depends largely upon the surface exposed to the reagent. Such factors as manner and rate of drying, density, degree of subdivision of the material, agitation of the dispersion mixture, as well as the nature of the fibrous material and the cuprammonium solution, must be taken into consideration. When these factors remain fairly constant the dispersibility of the cotton fiber in cuprammonium solution is, however, dependent upon the relative proportions of the many fiber components and the interference which certain of these components offer toward the dispersing agent.

The effect of various purification treatments upon the specific viscosity and calculated degree of polymerization of the developing cotton fiber is given in Tables II and III. Extraction with ammonium oxalate solution of cotton fibers previously extracted with water and alcohol-benzene in general lowers the specific viscosity to a slight extent. Further extraction with 1 per cent sodium hydroxide solution at 80° C. in an inert atmosphere continues to lower the specific viscosity (Table II). On the other hand, the direct treatment of the water and alcohol-benzene extracted developing cotton fibers with 1 per cent sodium hydroxide solution at 80° C. increases the specific viscosity of the resulting cuprammonium dispersions (Tables II and III). The alkali extraction thus seems to remove a component of the water and alcohol-benzene extracted cotton fibers which prevented the cuprammonium dispersions from attaining the maximum viscosity of which the system is capable. These results are also more easily explainable

<sup>5</sup> Cotton fiber pectic acid was removed or decomposed to the extent of 90 to 95 per cent by this procedure. Samples of citrus pectin similarly treated were decomposed to the extent of 80 to 90 per cent.

upon the basis of the dual structure of the cotton fiber than upon the single phase molecular concept.

In previous communications (5, 6, 7) the opinion has been expressed that the cuprammonium viscosity of cellulosic materials was not dependent upon any one of the many fiber components alone, but rather was dependent upon the colloidal system (cellulose particles-intercrystalline material) present in native fibers. As an extension of this point of view a study was made of the change in specific viscosity of cuprammonium dispersions of developing cotton fibers. The results obtained are given in Tables II and III. It can be seen that the specific viscosity of developing cotton fibers, extracted only with water and alcohol-benzene, increases until about the thirtieth to thirty-fifth day, after which the viscosity drops about 15 per cent, a value which remains constant until the boll opens. According to the classical correlation (26, p. 475-498), between specific viscosity and degree of polymerization,  $D.P. = \eta_{sp}/C \cdot K_m$  (see Section I of Experimental), which assumes that the viscosity is due only to the cellulose polymer  $(C_6H_{10}O_5)_x$ , this variation is due to a change in average length or degree of polymerization of the cellulose molecules. These values have been calculated using the value,  $K_m = 5 \times 10^{-4}$ , for the constant in the equation<sup>6</sup> (Tables II and III). Since it would hardly seem reasonable that the cellulose molecules would first increase in chain length and then decrease, as the fiber develops, the results seem to lend further support to the dual structure of the cotton fiber and of the resulting cuprammonium dispersions. That is to say, the interrelation between the crystalline cellulose and the intercrystalline fiber components varies as the fiber develops, the colloidal cuprammonium dispersions attaining a maximum viscosity between the twenty-fifth and thirty-fifth day of fiber development, thereafter undergoing a slight decrease.

Recently, Hess, Wergin, Kiessig, Engel, and Philippoff (18) presented results contrasting the calculated degree of polymerizations of cuprammonium dispersion of 10-day cotton fibers, before (1520) and after (1570) water extraction with similarly treated mature cotton fibers (1730 and 1810, respectively). The difference between the calculated degrees of polymerization is approximately 15 per cent. The values which have been obtained in the present investigation, Table II, for the 10-day and 55-day cotton fibers extracted with water and alcohol-benzene are 1966 and 2826, respectively, or a difference of approximately 30 per cent. However, the maximum degree of polymerization (27) obtained at 25 days (3364) differs by 41 per

<sup>6</sup> The value of  $K_m$  was determined at 20° C. with a specially prepared cuprammonium solution by Staudinger. The  $\eta_{sp}/C$  values of the present work were determined at 25° C. in the cuprammonium solution described above. The calculated D.P. values of Tables II and III are thus consistent approximations, which cannot be considered absolute until corrected for the effect of the change in temperature on the  $K_m$  value.

cent from that of the 10-day sample. Upon extrapolating to zero concentration, or infinite dilution, the  $\eta_{sp}/C$  values obtained at various concentrations for the 10- and 55-day cotton fibers, column 9, Table III, the  $\eta_{sp}/C$  values 0.589 and 1.080, respectively, are obtained. These values are equivalent to calculated degrees of polymerization of 1178 and 2168, respectively, or a difference of 45 per cent between the 10-day and mature cotton fibers. These results are summarized in Table VI.

Wergin (28), reporting the experimental work of Philippoff, had previously determined the calculated degree of polymerization and chain length of the mature cotton fiber from viscosity measurements. These workers found a maximum calculated degree of polymerization of 1840.

TABLE VI

COMPARISON OF THE CALCULATED DEGREE OF POLYMERIZATION AND AVERAGE LENGTH OF CELLULOSE UNITS OF 10-DAY AND MATURE COTTON FIBER DISPERSED IN CUPRAMMONIUM HYDROXIDE SOLUTION

Age	Calculated degree of polymerization,* $K_m = 5 \times 10^{-4}$		Calculated length of cellulose units*		X-ray diagram of coagulated material
	Present work	Obtained by Hess and Philippoff	Present work	Obtained by Hess and Philippoff	
10-day	1178	1570	0.59 $\mu$	0.78 $\mu$	Merc. cellulose
Mature	2168	1810	1.08 $\mu$	0.90 $\mu$	Merc. cellulose

$$* \text{ D.P.} = \frac{\eta_{sp}}{C \cdot K_m}; L = 5 \times \text{D.P.} \times \text{\AA} E.$$

and the cellulose chain length to be 0.92  $\mu$ , from the equation,  $L = 5 \cdot P \cdot \text{\AA} E$ , in which  $L$  is the length of the cellulose molecules,  $P$  is the calculated degree of polymerization, and  $\text{\AA} E$  the conversion factor of Angstrom units to microns. In the present work, the length of the cellulose molecule, calculated in a similar manner, has been found to be 1.08  $\mu$  (Table VI). Since, according to Wergin's microscopic observations, the cellulose particles are 0.25  $\mu$  in length, Philippoff was unable to explain how molecules 0.92  $\mu$  in length could exist in particles 0.25  $\mu$  in length, assuming of course that the particles disrupted in cuprammonium to a homogeneous molecular state. Cellulose molecules 1.08  $\mu$  in average chain length could originate from microscopic cellulose particles of the dimensions 1.1  $\times$  1.5  $\mu$ . From observations made on cuprammonium dispersions of purified cotton fibers (5, 6), however, the cellulose particle fiber component does not appear to be appreciably disrupted to submicroscopic dimensions. The assumption that heterogeneous fiber dispersions, which behave in a manner somewhat analogous to true molecular dispersions, are molecularly dispersed would thus appear questionable. The possibility that disruption of a part of the



total cellulose particles to submicroscopic dimensions in cuprammonium solution to cause the resulting heterogeneous dispersion to exhibit homogeneous molecular behavior is not excluded. Unbiased study of complex fiber systems composed of closely related carbohydrate materials which are apparently capable of dispersing to different states of molecular aggregation, will doubtless clarify the issue.

Since the cell wall of the young cotton fibers has been found to consist largely of pectic material, in addition to cellulose (10, 13, 20, 23), interest centers around the possible rôle of this substance in the development of the cotton fiber. As pointed out by Norman (20), pectic substance is the only constituent of the cell wall, besides cellulose, which may be estimated with accuracy by a relatively simple gravimetric process. The procedure is based on the insolubility of calcium pectate and the fact that this substance has a relatively constant composition. The greatest disadvantage of the method lies in the fact that the separate pectic constituents, protopectin and pectin, can only be expressed in terms of the calcium pectate formed from them. Until the exact nature of the pectic constituents is known, however, no alternative is possible. The use of the calcium pectate number introduces little error inasmuch as the molecular weight of partially esterified pectin is not very different from that of the calcium salt. As may be seen in Table IV, the calcium pectate number of 6.5 of 10-day dry crude cotton fiber decreases very rapidly as the fiber develops so that after about the fortieth day it is slightly less than one. Comparison of columns 4 and 5, Table IV, shows that the ratio of dry crude cotton fiber to calcium pectate per boll changes from 5 to 1 in 10-day cotton fiber to about 100 to 1 in 50-day cotton fiber. In column 5, Table IV, it will be noted that the weight of calcium pectate obtained per boll increases until about the thirtieth day, thereafter remaining constant. There would thus appear to be an accumulation of pectic substances in the fiber as development proceeds. The calcium pectate number calculated on the basis of the dry crude cotton fiber after extraction with water is much higher than that of the dry crude fiber containing all of its many constituents, column 7. When these values are compared with the calcium pectate number of a series of developing cotton fibers grown in 1938, but extracted with water and alcohol-benzene prior to the calcium pectate determinations, similar results are obtained (Table IV). From this the inference may be drawn that practically all of the pectic material of the cotton fiber is in a form insoluble in water.

Comparison of the trends shown toward an increase in viscosity of cuprammonium dispersions of developing cotton fibers, with the decrease in calcium pectate number, would seem to indicate that neither free nor combined cotton fiber pectic acid is directly related to the viscosity-producing components of the fiber system. Attention has previously been called to the apparent interference of this fiber component to dispersion of cotton

fibers in cuprammonium solution. The importance of the proven existence of at least one polyuronide in cotton fibers lies in the possibility that the intercrystalline, amorphous, fiber phase is composed of various molecular modifications intermediate between those of these substances and the true cellulose molecule. An entirely new approach is thus brought to the study of the chemical and physical behavior of natural fibers.

The variation of the hygroscopic moisture content of the developing cotton fiber is to a large extent dependent upon the nature of the fiber components (Table IV). The high moisture content of young cotton fibers is due, principally, to the presence of sugars and other water-soluble materials as shown upon comparing columns 2 and 3, Table IV. The pectic materials may also cause a high moisture content in the young fibers (column 4, Table IV). After extraction with sodium hydroxide solution any hemicellulosic material remaining has very little, if any, hygroscopic properties.

#### SUMMARY

1. The dispersibility of cotton fibers at various stages of development in cuprammonium hydroxide solution is dependent both upon the age of the fiber and the purification treatment to which it has been subjected.

2. The specific viscosities of cuprammonium dispersions of developing cotton fibers increase until a maximum is reached at 30 to 35 days, after which a slight decrease occurs.

3. The specific viscosity of cuprammonium dispersions of developing cotton fibers after various purification treatments can be arranged according to decreasing values as follows: fibers extracted with (A) water, alcohol-benzene and 1 per cent sodium hydroxide solution at 80° C. for four hours in a nitrogen atmosphere; (B) water and alcohol-benzene; (C) similar to (B) plus treatment with 0.5 per cent ammonium oxalate solution at 80° C.; and (D) similar to (C) plus treatment with 1 per cent sodium hydroxide solution at 80° C. for four hours in a nitrogen atmosphere.

4. From (2) and (3) it appears that the relation between the viscosity-producing components of the cotton fiber system (cellulose particles-intercrystalline material) varies both as the fiber develops and as a result of various purification treatments at each stage of development.

5. The high calcium pectate numbers of young developing cotton fibers decrease rapidly from the tenth to thirtieth day, thereafter remaining practically constant until the boll opens.

6. The high hygroscopic moisture content of raw cotton fibers at various stages of development is principally due to the presence of water-soluble carbohydrates and pectic material.

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# OXYGEN INTAKE AND CARBON DIOXIDE OUTPUT OF GLADIOLUS CORMS AFTER STORAGE UNDER CON- DITIONS WHICH PROLONG THE REST PERIOD

NORWOOD C. THORNTON AND F. E. DENNY

Previous reports (2, 3) have shown, first, that the dormant period of the corms of many varieties of gladiolus could be prolonged for many months, or more than a year, by merely planting the freshly-harvested corms in moist soil, and storing them at room temperature; secondly, that the carbon dioxide output of such corms, although very low during the first few hours after removal from the soil, increased many-fold during the next 20 to 40 hours, and then receded rapidly within 4 days until the original low value was approached.

These changes in respiration rate were obtained without a change in temperature, and corms having once been used in a respiration test remained dormant when replanted in soil, and furnished a second respiration curve similar to the first one, provided the second period in the soil was for a period as long as three months.

The present experiments were undertaken, first, to determine the oxygen intake under these conditions, and to compare the volumes of oxygen taken in and carbon dioxide released at intervals during the period of these progressive changes in respiration; secondly, to estimate the amount of carbon dioxide held within the corm tissues at the time of removal of stored corms from the soil, and at intervals during the respiration tests, in order to determine whether the release of this internal gas was a factor in the measurements of carbon dioxide output.

The results show that the output of carbon dioxide is accompanied by an intake of oxygen, and further, that in the first few hours after the removal of the corms from the soil, in the phase when the respiration is increasing to a maximum, the volume of oxygen absorbed is much greater than the volume of carbon dioxide given off, usually two to three times as great. In the phase of the falling respiration, in the hours and days at or after the maximum rate, the volumes of the two gases are equal.

Only small amounts of carbon dioxide were found in the internal gas which was extracted from the corms at the time of removal from the soil, indicating that the increase in CO<sub>2</sub> production in the period after removal of the corms from the soil was not due to the release of CO<sub>2</sub> previously formed and absorbed by the tissue.

## MATERIAL AND METHODS

*Corms.* Corms from the harvests of 1938 and 1939 were used in the tests. Approximately one week after the corms were lifted in October of each

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year they were peeled and replanted in moist soil in flats which were stored in tiers at room temperature. The soil in the flats was kept moist by frequent examination of the soil condition. The results reported in Table I were obtained in July 1939 with corms of the 1938 crop, and the tests described in Tables II and III were carried out in April 1940 and included corms from the harvests of both 1938 and 1939. At the time of the respiration measurements, therefore, the corms had been held in soil from 6 to 18 months. They were still quite dormant, however, and when replanted in soil at the expiration of these tests did not germinate, at least not for several weeks thereafter, and in the case of corms of the varieties *Senorita* and *Purple Glory* not for several months.

*Gas analyses.* The rate of respiration was determined by gas analysis of the atmosphere of the container in which the bulbs were held. For experiments that were planned to be carried out over a period of several days with the same lot of corms, the container used was one with a volume of approximately 8 liters. The proportion of air volume to corm volume in most of such cases was approximately 10, which permitted respiration to occur for several hours without unduly reducing the  $O_2$  content or increasing the  $CO_2$  content, but at the same time permitted sufficient change in the proportion of these two gasses to give satisfactory measurements by gas analysis. The details of sealing the containers and of removing and analyzing the sample of air are shown in a previous report (5). Samples of air were taken at such intervals as would prevent any undue accumulation of  $CO_2$  or reduction in  $O_2$  content. In special tests the oxygen content of the atmosphere about the corms was allowed to be reduced to about 17 per cent and the carbon dioxide content to increase to about 4 per cent during a respiration test, but in no case did this give any different results from those tests run for short periods where the oxygen content was maintained at or near the normal level and very little carbon dioxide was allowed to accumulate. After a sample of air was removed for gas analysis a current of  $CO_2$ -free air was drawn through the containers in order to replace the partially respired air within the container by fresh air. This precaution was exercised after every 4-, 6-, 10-, or 24-hour period during tests of long duration, the interval depending on the rate of respiration at the time.

In experiments for short periods smaller containers were used and these were filled with corms in order to decrease as much as possible the ratio of air volume to corm volume. The details of such procedures are described in the text relating to the results obtained by experiments of short duration.

*Internal gas.* The determination of the internal gas of gladiolus corms was made with the Magness apparatus (4) by placing each sample of four corms in the chamber and subjecting them to a Torricellian vacuum after all the free air had first been driven from the chamber. The composition of the extracted gas was determined by gas analyses.

## EXPERIMENTAL RESULTS

MEASUREMENTS OVER AN EXTENDED PERIOD AFTER REMOVAL OF  
CORMS FROM THE SOIL

The results of respiration tests on corms of the 1938 crop stored in moist soil from October 1938 until July 1939 are shown in Table I. The volumes of  $\text{CO}_2$  output and  $\text{O}_2$  intake are shown in columns 3 and 4. It is seen that in the early periods after removal from the soil the volume of  $\text{O}_2$  taken in is much larger than the volume of  $\text{CO}_2$  eliminated. In some cases, as with Dr. F. E. Bennett, Scarlet Princeps, Purple Glory, the difference in volumes of the two gases is greatest in the period during the first four hours after removal from the soil; with Odin the difference did not appear until after the fourth hour; with Halley and Senorita it was maintained up to the tenth hour. In all cases beginning at least with the 19th hour the volumes of  $\text{CO}_2$  output and  $\text{O}_2$  intake became equal, and this equality was maintained for as long as 14 days thereafter.

The data for both  $\text{CO}_2$  and  $\text{O}_2$  show that after a maximum rate of respiration was reached at about 20 to 30 hours there was a gradual decline to a low value after about 200 to 300 hours. This final low value in the case of  $\text{CO}_2$  was about the same as the early value obtained in the 0-4 hour measurement, but the final  $\text{O}_2$  value was usually lower than the  $\text{O}_2$  value obtained in the earliest stage.

When there is no entry in column 2, Table I, to account for all of the time period, as for example, during the hours 62 to 321 for Dr. F. E. Bennett, this does not mean that no analyses were obtained during such intervals. Measurements were made and showed equal volumes of  $\text{CO}_2$  and  $\text{O}_2$  for each interval. They were omitted from Table I merely to bring the table within bounds for publication.

MEASUREMENTS OVER SHORT INTERVALS IN THE EARLY PERIOD  
AFTER REMOVAL OF CORMS FROM THE SOIL

Since the results showed that the divergence in the volumes of the  $\text{CO}_2$  output and  $\text{O}_2$  intake occurred only during the first few hours after removal of the bulbs from the soil, attention was turned toward obtaining measurements at short intervals beginning immediately after removal. This required that the volume of air surrounding the corms be small in comparison to the weight of the corms, in order that a large percentage change in the amounts of  $\text{O}_2$  and  $\text{CO}_2$  in the air surrounding the corms could occur during short intervals.

For this series of tests, glass desiccators of the proper size to be filled completely with the sample of corms, were selected in each case. After the corms were in place a current of air freed of  $\text{CO}_2$  by passage through soda-lime tubes was drawn through the desiccator for a few minutes. Then a

TABLE I  
OXYGEN INTAKE AND CARBON DIOXIDE OUTPUT OF GLADIOLUS CORMS AT VARIOUS  
PERIODS AFTER REMOVAL FROM SOIL

Variety	Hours after removal from soil*	Respiration (cc. per kg. per hour)	
		CO <sub>2</sub> output	O <sub>2</sub> intake
Dr. F. E. Bennett	0- 4	4	14
	4- 10	18	21
	10- 19	28	28
	19- 29	31	31
	29- 39	27	27
	39- 62	15	15
	321-345	4	4
Halley	4- 10	13	33
	10- 19	52	52
	19- 29	51	51
	29- 39	35	35
	39- 62	17	17
	321-345	6	6
Odin	0- 4	6	6
	4- 10	15	26
	10- 19	46	46
	19- 29	37	37
	29- 39	20	20
	39- 62	11	11
	321-345	6	6
Scarlet Princess	0- 4	**	16
	4- 11	14	14
	11- 23	16	22
	23- 30	14	14
	30- 48	12	12
	221-291	4	4
Purple Glory	0- 4	3	6
	4- 10	16	18
	10- 19	17	17
	19- 29	20	20
	29- 39	20	20
	39- 62	14	14
	321-345	4	4
Senorita	0- 4	3	10
	4- 10	7	14
	10- 19	14	14
	19- 29	15	15
	29- 39	15	15
	39- 62	8	8
	321-345	2	2

\* Fresh CO<sub>2</sub>-free air drawn through the respiration chamber after each test period.

\*\* The rate of carbon dioxide production by 400 g. of corms during the first four hours of this experiment was so low that it could not be determined by an Orsat gas analysis apparatus, but the rate of respiration was not zero since carbon dioxide could be shown to be present by absorption in Ba(OH)<sub>2</sub>.



sample of the moving air stream was taken at the exit by a gas burette for analysis (to show the gas composition at the start); inlet and outlet tubes were closed; after a period of time, the gas burette was again attached and after pumping the gas several times by alternately lowering and raising the burette, a sample was removed and analyzed. The effective volume of air in the desiccator containing the corms was determined at the end of the test by the volume of water needed to fill the apparatus, the corms remaining in place.

TABLE II

RESPIRATION OF GLADIOLUS CORMS AT SHORT INTERVALS SOON AFTER REMOVAL OF CORMS FROM SOIL

(ALIQUOT OF AIR REMOVED FROM CONTAINER BY SUCTION WITH A GAS BURETTE)

Variety	Weight of sample of bulbs, g.*	Volume of air surrounding bulbs in the container, cc.	Hours after removal from soil**	Respiration (cc. per kg. per hr.)	
				CO <sub>2</sub> output	O <sub>2</sub> intake
Mixed	1130	1360	0-2	2	5
			3-6	4	8
			7-11	6	18
Odin	980	1680	0-3.5	3	12
			4-6	4	15
			8-12	9	36
Senorita	1060	1490	2.5-4.5	4	8
			5-7	6	11
Purple Glory	1030	1540	0-2	3	5
			2-4	12	11
			5.75-7.75	9	14
Salmon Star	1060	1490	0-2	3	4
			2.5-4.5	6	16
			5.5-7.5	7	13
Dr. F. E. Bennett	960	1520	0-2	4	4
			3-5	6	14
Minuet	1140	1500	0-2	4	4
			3-5	5	9

\* Corms packed closely in desiccators so as to occupy nearly all of the space.

\*\* Fresh CO<sub>2</sub>-free air drawn through the respiration chamber after each test period.

The results of experiments by this method are shown in Table II. The values for the O<sub>2</sub> intake are higher than those for CO<sub>2</sub> output in all intervals for all varieties except in the earliest period for Dr. F. E. Bennett and Minuet, and in the intermediate period for Purple Glory. The ratio of O<sub>2</sub> to CO<sub>2</sub> is about 2. There is some indication that the highest ratio is not in the earliest stage, but that it occurs somewhat later. The values for both CO<sub>2</sub> and O<sub>2</sub> agree in showing that the respiration rate is low in the early period after removal of the corms from the soil and that this low value is followed by an increase within a few hours thereafter.

In order to obtain an even greater change in volume percentage of carbon dioxide and oxygen in the respiration chamber during short periods, a respiration vessel consisting of a test tube 5 cm. in diameter with an opening at the bottom to aid in displacing the air from the apparatus was used. By selecting corms of sufficient diameter to fit snugly into this respiration chamber and flat enough to lie closely together, a still further reduction in the volume of air in proportion to weight of bulbs was obtained. After the corms were in place carbon dioxide-free air was drawn rapidly through this respiration chamber for a few minutes, then a sample of the moving air stream at the exit of the chamber was taken for analysis following which

TABLE III  
RESPIRATION OF GLADIOLUS CORMS AT SHORT INTERVALS SOON AFTER  
REMOVAL FROM SOIL  
(ALIQOT OF AIR REMOVED BY LIQUID DISPLACEMENT)

Variety	Weight of sample, g.	Volume of air, cc.	Period after removal from soil, hours	Displacing liquid	Respiration (cc. per kg. per hour)	
					CO <sub>2</sub> output	O <sub>2</sub> intake
Golden Measure	230	235	0-2	NaCl	4	6
			4.75-6.75	Hg	6	12
Giant Nymph	280	160	0-2	NaCl	2	6
			4.75-6.75	Hg	8	19
Odin	308	150	0-2	NaCl	2	3
			2.75-4.75	Hg	3	6
			5-7	Hg	7	12
Scarlet Princeps	300	155	0-2	NaCl	2	3
			2.25-4.25	NaCl	2	7
			4.5-6.5	NaCl	5	13

the inlet and exit tubes were closed. After a respiration period of two hours the gas sample surrounding the corms was displaced into a gas burette either by means of mercury, or by a 22 per cent aqueous solution of sodium chloride, a liquid recommended by Dennis and Nichols (1) as having a minimum solubility for both O<sub>2</sub> and CO<sub>2</sub>. The carbon dioxide output and oxygen intake were determined by differences in the analyses at the beginning and end of the test period (Table III). For subsequent tests the corms were removed from the chamber, washed, dried, and replaced for a second or third measurement. The effective air volume was measured by adding water to fill the apparatus.

In these respiration tests made at an early stage after removal from the soil, as shown in Table III, the rate of intake of oxygen always exceeded

the rate of production of carbon dioxide in each interval for which measurements were made. The oxygen intake exceeded the carbon dioxide output by amounts varying from 150 to 350 per cent. There is evidence in Table III which indicates that the excess of  $O_2$  intake over  $CO_2$  output is less at the earliest period (0-2 hrs.) than at a later time (4.5-6.5, or 5-7 hrs., etc.).

#### INTERNAL GAS

In order to determine whether escape of previously absorbed carbon dioxide, held in the tissues and released from the corms when they were removed from the soil, was a factor in the  $CO_2$ -output measurements, the internal gas was extracted from corms and analyzed.

Corms were removed from the soil in the usual way and a sub-sample consisting of two lots of four corms each was taken for the removal of internal gas to show the condition at the time of removal from the soil. The remainder of the sample of the corms was placed in the respiration chambers and a current of  $CO_2$ -free air was drawn through continuously. At certain intervals thereafter duplicate sub-samples of four corms each were removed to show the internal gas at these stages of the respiration curve.

For the removal of the internal gas the bulbs were placed in the Magness (4) apparatus and exposed to a Torricellian vacuum for two minutes. Various procedures for extracting the air and longer periods of vacuum suction were tested but no larger yields of internal gas were obtained with them than were obtained by using whole corms and exposing them to the vacuum action for two minutes.

The amount of gas extracted was small, being only approximately 0.04 cc. per gram of corm tissue. Gas analysis showed the percentage composition indicated by the curves in Figure 1, in which the results are expressed as per cent of the volume of the extracted air.

At the time of removal from the soil the corms contained only a small volume of total gas extractable by this procedure, and the curves in Figure 1 show that of this total gas only a small percentage (3.8 per cent) was  $CO_2$ ; the oxygen content at this time, however, was high (18.9 per cent).

The sample at the 55th hour after removal from the soil (at a time when the respiration of the corms was very high) showed that the  $CO_2$  content of the internal gas was high (30 per cent), and the oxygen was low (7 per cent). It is possible that, at this period when the respiration was high, the excess of internal  $CO_2$  and deficiency of oxygen may have limited the respiration to a rate lower than that which the tissue could have maintained under more favorable conditions with respect to these two gases.

Subsequently, as shown by the sampling periods at the 125th and 192nd hours (at a time when the respiration rate was receding toward the low rate characteristic of the corms at the time of their original removal from

the soil), the  $\text{CO}_2$  was decreasing and the  $\text{O}_2$  was increasing, until, at the final sampling, the internal gases showed approximately the same composition as had been found at the original sampling at the time of removal from the soil.

These analyses of the internal gas show that the increase in  $\text{CO}_2$  output which occurs shortly after the removal of corms from the soil cannot be due to the release of a previously accumulated quantity of  $\text{CO}_2$ . There is not

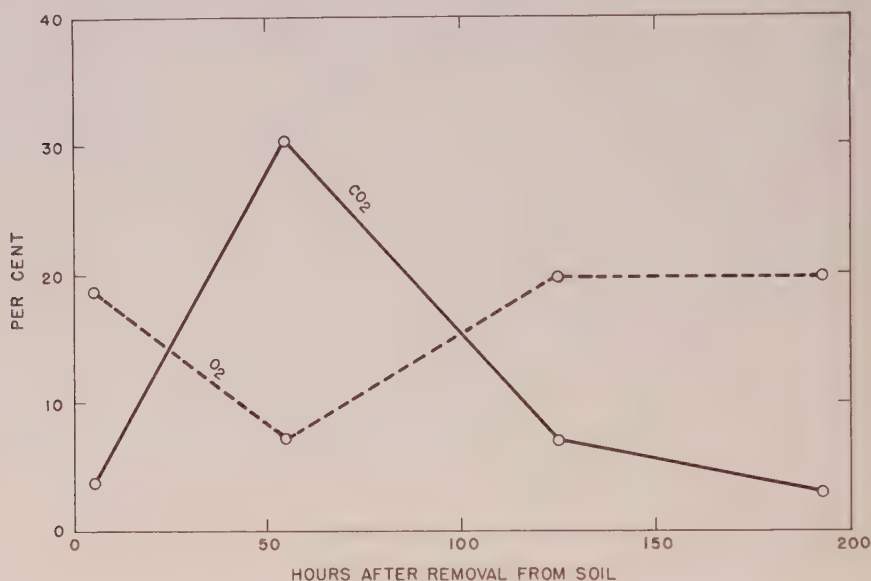


FIGURE 1. Composition of internal gas extracted from gladiolus corms by a Torricellian vacuum; expressed as per cent by volume of the total extractable gas; variety Dr. F. E. Bennett.

enough total internal gas, and the  $\text{CO}_2$  content of the gas that does occur is too low. In fact, these curves for the internal percentages of  $\text{O}_2$  and  $\text{CO}_2$  correlate very well with, and are consistent with, the respiration measurements based on the  $\text{O}_2$  intake and  $\text{CO}_2$  output at various stages after removal of corms from the soil.

#### COMPOSITION OF AIR IN SOIL

An analysis was made of the soil air surrounding the stored corms for comparison with that of air in soil without corms. Two flats (45 cm.  $\times$  32 cm.  $\times$  7 cm.) were filled three-quarters full with soil; in one flat 25 dormant gladiolus corms of the type used in these experiments were planted, while in the other flat no corms were placed; these two flats were kept well sup-



plied with water during a three months' storage period at room temperature. Air samples were removed from the soil of these two flats in the following manner: a galvanized iron plate (equipped in the center with a rubber stopper and glass exit tube) of the proper size to fit into the flat and cover the soil with only narrow margins between the plate and the sides of the flat, was placed on top of the soil; the margins between plate and flat were now sealed with clay and this was painted with melted paraffin; the sides and bottom of the flat were covered with gummed paper and the entire flat was placed in a shallow pan containing a layer of melted paraffin; the surfaces of the paper were painted with melted paraffin. After the paraffin had solidified a gas burette was attached to the exit tube, and after alternately lowering and raising the leveling bulb to mix the air in the soil, a sample of air was removed for analysis.

The results of the analyses show that the soil air from flats in which the corms were stored contained 0.6 per cent carbon dioxide (by volume) and 20.0 per cent oxygen as compared with 0.2 per cent carbon dioxide and 20.4 per cent oxygen in the soil air from the flats in which no bulbs were stored. Whether such a difference in the composition of the soil air has an effect upon the dormancy of the corms is not known, but that it does not lead to an accumulation of  $\text{CO}_2$  in the stored corms is shown by the results of the analyses of the internal gas of the corms, as described in a preceding section of this paper.

#### SUMMARY

Gladiolus corms, held for 6 to 18 months in the dormant condition by replanting the freshly-harvested corms in moist soil and storing them at room temperature, showed a low rate of carbon dioxide production for the first few hours after removal from the soil; this rate increased to a maximum at about the 20th to 30th hour, and then decreased to the original low rate, which was then maintained. Such corms when again planted in the soil continued for many weeks, or even months, depending upon the variety, in the dormant condition.

During the first few hours after removal from the soil the volume of oxygen taken in was much greater than the volume of carbon dioxide eliminated, usually two to three times as great. At about the time of the maximum rate of respiration the oxygen intake and carbon dioxide output became equal in volume, and this equality was maintained at all points in the falling phase of the respiration curve.

Only small amounts of gas, about 0.04 cc. per gram of tissue, could be extracted from the corms by a Torricellian vacuum. Analyses of this internal gas extracted from corms at the time of their removal from the soil showed low values for  $\text{CO}_2$  (3.8 per cent, by volume) and high values for  $\text{O}_2$  (18.0 per cent); at the time of the high respiration rate the internal gas

showed high values for  $\text{CO}_2$  (30 per cent) and low values for  $\text{O}_2$  (7 per cent); in the late phase of the respiration curve when the rate had receded to low values, the composition of the internal gas with respect to  $\text{CO}_2$  and  $\text{O}_2$  was similar to that shown by the corms at the time of their removal from the soil.

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## PROPAGATION OF *EPIGAEA REPENS* L. II. THE ENDOPHYTIC FUNGUS

FLORENCE L. BARROWS

A study of propagation of *Epigaea repens* L. was begun in 1933 to determine the conditions for rooting cuttings, and for seed germination. This phase of the problem has already been discussed (3). At the same time a survey was begun of the distribution of the fungus in the plant, and the way in which it becomes established in the seedlings. Isolations of the fungus were made (3, p. 95).

As previously mentioned in Part I (3, p. 81), Coville (9, p. 711) made the important discovery that the trailing arbutus, *Epigaea repens*, has a symbiotic root fungus, "similar to the beneficial and probably indispensable root fungus of the blueberry." Applying the same system of culture which had proved successful with the blueberry and its mycorrhizal fungus, Coville (9) was the first to report success in raising trailing arbutus from seed.

Councilman (7) studied the root system of *Epigaea repens* and its relation to the fungi of the humus. His figures bring out clearly the fibrous nature of the root system and also the mycorrhizal fungus coiled in the epidermal cells. His material came from Maine, Massachusetts, and Maryland. The fungus was apparently always the same. A short later paper (8, p. 362) likened the fungus coil to a "kidney glomerulus." He considered the endophytic fungus a symbiont and an aid in nutrition of the plant which he says "lays up no food supply."

Lemmon (12, p. 101) says "A healthy specimen of *Epigaea* has a surprisingly large and very finely fibrous root system which is well supplied with a minute mycorrhizal fungus whose existence in an active state is essential to the plant's well-being. It is next to impossible to dig up such a plant without breaking a considerable number of the fragile feeding root-lets or shifting the soil about them enough to dislodge the mycorrhiza—or both. As a result, the natural balance is destroyed and the plant customarily dies before it is restored."

Although the presence of an endophytic fungus in roots of *Epigaea repens* has been mentioned by several authors, no explanation has been made as to how the fungus becomes established in the seedlings. In 1935, while collecting fruits from wild plants, the presence of the fungus was observed in the fruits and on the seed coats (3, p. 89). The present report covers observations on the presence of the endophytic fungus not only on the fruits, but also in the flowers, on the pollen and on the young ovules

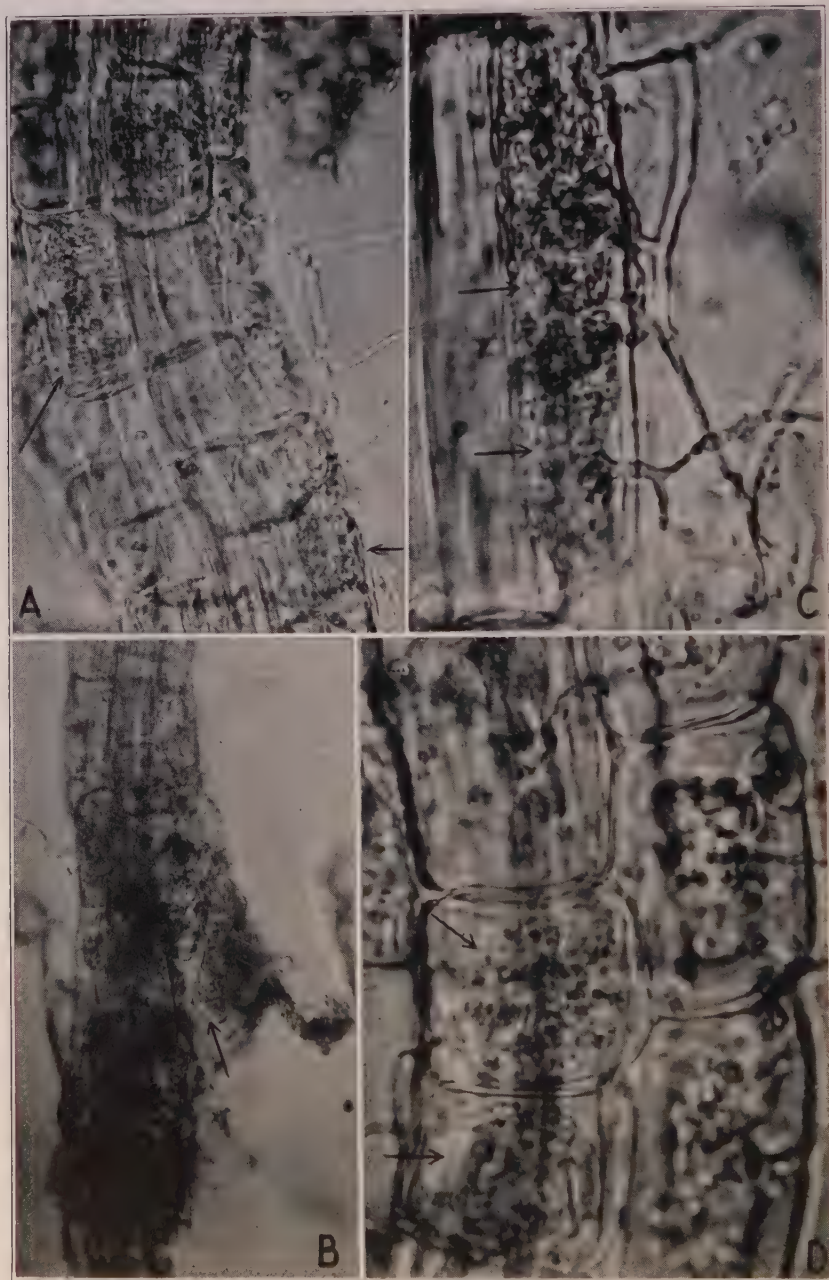


FIGURE 1. Endophytic fungus coils (see examples shown by arrows in each of A, B, C, D) in root epidermis of *Epigaea repens*. A. Two-year seedling ( $\times 440$ ). B. Epidermis separated from central cylinder ( $\times$  approx. 190). C, D. Coils with hyphae extending through epidermal cell walls ( $\times 850$ ).



enclosed within the carpels, and in the stems of the plants. The fungus has been isolated from various parts of the plant. Fungus inoculation tests have been made using soil with broken arbutus roots known to contain the endophyte.

#### DISTRIBUTION OF THE FUNGUS

*Cuttings.* Cuttings taken August 29, 1933, at Union, Connecticut, were previously described and illustrated (3, p. 82, Fig. 1 C to F). The prostrate stem of *Epigaea repens* shows the presence of the fungus in cortex, xylem, and pith in cross sections. The iodine and potassium iodide test revealed the presence of numerous starch grains in these woody stems. The starch was abundant in the pith and in the medullary ray cells between the xylem cells. The same positive reaction for starch was found in numerous samples collected in Connecticut; in some plants from Exeter, New Hampshire which had 4 and 5 annular rings; and also in 1- and 2-year stems of cut flowers obtained from New York, but which probably originated in the Carolinas. The iodine test indicated the accumulation of starch in *Epigaea repens*. These stems also contained an endophytic fungus within the cortex, xylem, and pith. The relation between starch storage and the endophyte needs further investigation.

Microscopic examination of roots formed on cuttings revealed the typical endophytic fungus coiled in the epidermal cells. The roots of this species, like other members of the Ericaceae, lack root hairs. These roots, however, cling very tenaciously to humus in the soil or rooting media, especially to particles of peat (3, Fig. 1 A) or leaf mold. The connection is more firmly made through the attachment of fungal hyphae which connect the endophytic coils within the epidermal cells with external mycelia over the surface of the root, and the mycelia in the humus of the soil (Fig. 1). In fact this attachment is so firm that unless extreme care is used in transplanting seedlings or cuttings, the epidermis containing the fungus coils is stripped off (Fig. 1 B). If care is not used, sometimes the whole epidermis containing the endophyte may pull away leaving only the central cylinder of the root. Placing forceps or some other instrument well down beneath the plant helps to lift the whole root system, and causes less injury.

The endophytic fungus is found close to the meristem, but not at the growing point of the roots. The dividing cells seem to keep a little ahead of the fungus. The denser coils are found back from the apex of the root and are irregularly scattered, sometimes several adjacent cells being entirely filled with the fungus (Fig. 1 A, D).

*Collected plants.* Abundant fungus coils typical of the endophyte were found in *Epigaea* plants collected from Union, Connecticut on August 29, 1933 (3, p. 84). Fungus coils were also numerous in roots of specimens collected from March 30 to November 25, 1934 in soils where the acidity ranged from pH 7.67 to 5.40 (3, p. 93, Table III). The same type of endophytic

fungus was present in *Epigaea* sent from Exeter, New Hampshire, as well as in two seedlings purchased from New Canaan, Connecticut, in the fall of 1935. It was also found in stems of two bouquets of cut flowers purchased from New York City florists in the springs of 1935 and 1936.

*Flowers, fruits, and seeds.* The mycorrhizal fungus was observed on and within the flowers of arbutus. Unopened anthers of buds contained coils of the fine hyphae in and on the pollen tetrads. The fungus was also inside the ovaries over the surface of the ovules in the carpels at or soon after fertilization. Slender hyaline hyphae typical of the endophytic fungus were present on and in the sticky white pulp of fruits as they matured and opened. Examination of mature but unopened fruits under aseptic conditions revealed the same type of delicate hyphae inside the carpels and on the seed surfaces. The endophytic fungus was found in practically every one of the 155 fruits collected in June, 1935, and also in the 1936 and later crops. The fungus seems to be normally present within the flower and fruit and on the seed coat.

Fruits were illustrated by Burnham (6) and Fletcher (11), and seeds were accurately described by Burnham (6). Stevens (14) and Andrews (1) reported on fertilization and development of fruit and seeds, but no mention of the presence of a fungus on fruit or seeds was made by any of these authors.

*Infection of seedlings.* Observations made during the progress of seed germination indicated that the fungus usually penetrated the hypocotyl soon after it emerged from the micropyle. The presence of the fungus on the seed coat enables it to become established in the seedling soon after rupture of the seed coat. This may be correlated with better germination and growth from seeds planted soon after ripening. Normal and vigorous seedlings were always found to contain the fungus. Stunted or abnormal seedlings were found to have very little of the fungus or lacked it entirely.

The typical fungus was found in the stem and roots of cuttings and in collected plants; on the flowers, pollen and ovules, seeds, and in the fruits from both wild and propagated plants, and on the surface of their seeds; and in seedlings raised from these seeds (Fig. 1 A, C, and D.)

#### ISOLATION OF THE ENDOPHYTIC FUNGUS

The endophytic fungus was isolated and grew readily on potato dextrose agar. Coils in roots were studied microscopically in distilled water on sterile slides. When left in a sterile moist chamber for a day or two, these coils frequently burst the epidermal cell walls and developed hyphae which grew out into the water. Such cultures transferred to flasks of sterile potato dextrose agar or 2 per cent maltose, soon developed a mycelium forming a white cottony mass, often slightly pinkish in tint. Flaming roots to sterilize

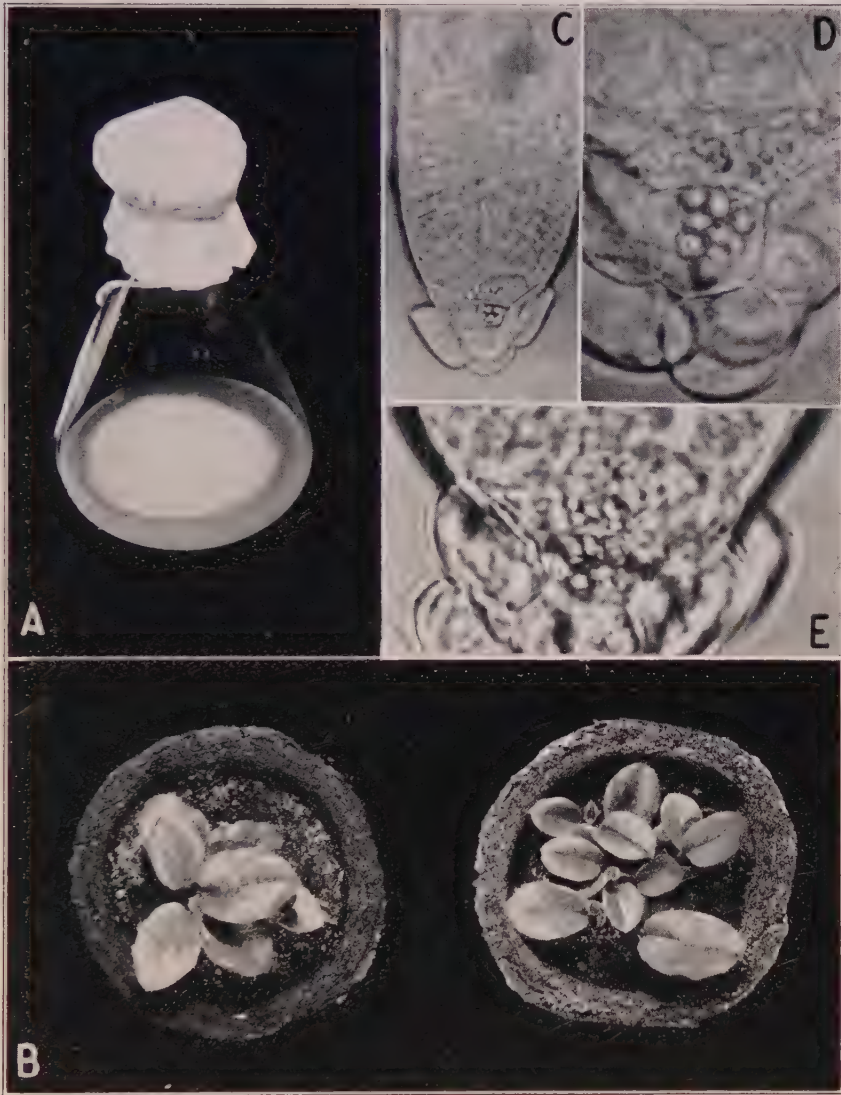


FIGURE 2. A. Endophytic fungus isolated from fruit of *Epigaea repens* L.; 3-day pure culture on potato dextrose agar. B. One-year-old seedlings in pressed peat pots. Roots of such seedlings contain the endophytic fungus ( $\times 0.47$ ). C. Living chromosomes in root tip of *E. repens* ( $\times 437$ ), and D. ( $\times 874$ ). E. in *E. asiatica* ( $\times 437$  enlarged to 874).



them was almost sure to kill the fungus, which is covered only by a thin epidermal wall of the root cell.

Later, however, it was found possible to sterilize roots according to Davies' (10) method, which was a quicker and more reliable procedure. By immersing the roots in a solution of 1 per cent silver nitrate and then in 0.7 per cent sodium chloride until no more silver chloride was formed and rinsing thoroughly in sterile distilled water before transferring to potato dextrose agar under aseptic conditions, pure cultures were obtained. Isolations were made from roots of both cuttings and collected plants. Transfers were made directly from the pulp of aseptically dissected fruits (Fig. 2 A) and from seeds in such fruits. Isolations were made from roots of seedlings (Fig. 2 B) in the same way as from the roots of cuttings or roots of collected plants. Various fungi, such as *Mucor*, *Penicillium*, and *Alternaria*, may be present on the surface of roots of *Epigaea repens*. But the endophytic fungus within the root tissue always seemed to be the same in plants from different sources and at different seasons of the year.

#### THE FUNGUS IN PURE CULTURE

*Macroscopic appearance.* The fungus grew readily on potato dextrose agar or in 2 per cent maltose. On the solid medium a cottony white aerial mycelium developed, often with a pinkish tint (Fig. 2 A). In 2 per cent maltose the mycelium frequently grew submerged and became gelatinous developing a deeper pink to rose color. In very old cultures this color might darken to a purplish-red or red-brown. The color diffused out into both agar and maltose. Rapidly growing young cultures were usually lighter in color than older ones.

*Microscopic structure.* The color of cultures in maltose was found to be in the wall of the hyphae. The color might be uniform throughout the wall or deposited as granules. Although no pH tests were made on aged cultures it is possible that color changes may have been associated with changes in acidity. Bessey (4) found that color changes in *Fusarium* were dependent upon acidity of media.

The hyphae of the endophytic mycorrhizal fungus as grown in pure culture are thin-walled and slender. Cross walls are formed in larger and older hyphae. The structure is similar to that of the endophyte isolated by Bouwens (5) from the quince, *Cydonia vulgaris*.

*Reproduction.* Aerial mycelia form numerous conidia. Chlamydospores, both terminal and intercalary, were frequently found in submerged mycelia in maltose and on potato dextrose agar in the older and somewhat dried cultures. These closely resembled the chlamydospores found in and on the roots containing mycorrhizae. The fungus in culture can survive weeks or months of drying and when transferred to fresh media develops actively growing mycelia. The growth of the fungus in pure culture at room tem-



perature is more active and vigorous during the spring months than during the mid-winter. This is similar to the vigor of the endophyte as found in the roots of *Epigaea*. The life history of the endophyte needs further investigation for identification.

#### SOIL INOCULATION

Two seedlings, which had been germinated by the Institute Seed Laboratory in 1932, were found on October 30, 1934 to be growing in soil with pH values of 5.92 and 5.28 (3, p. 94). The plants were small and the leaves were dwarfed, while the roots contained little or no fungus. A mixture of peat moss and sand in which cuttings had been rooted, and which was known to contain broken roots enclosing fungus coils of the endophyte, was added to the surface of the pot containing the less sturdy plant. The other plant served as a control. More soil with the fungus was added on January 3, 1935, by which time new shoot growth had started on the previously treated plant. By mid-September the control plant was dead, but the treated plant had developed larger leaves and a cluster of flower buds, which were later used as the pollen parent in a number of crosses. The typical endophytic fungus was isolated from the roots of this plant on September 15, 1935. Although only two plants were available at the time of this test, the development after inoculation with soil containing the fungus, and the difference in general growth and size of leaves, suggested the value of using soil known to contain the endophytic fungus. This was subsequently confirmed by treating four out of six 1935 cuttings on March 24, 1936. By May 11, long shoots of new growth had developed on the treated plants and dense coils of fungus were present in the roots. Much less growth was made by the controls in which no fungus coils were found in the roots examined.

When seedlings developed unevenly, the roots of the stunted plants were often found to contain only a small amount of the fungus or to lack it entirely. When such seedlings were repotted in soil known to contain the endophyte, they usually responded by renewed growth. After finding that such backward plants responded favorably to the soil inoculation treatment, it was made a regular practice when transplanting seedlings to add some soil in which cuttings or seedlings had already been successfully grown. The soil containing the endophytic fungus was either sprinkled over the surface or mixed with the upper layer. This usually proved a simple but efficacious means of securing good growth of seedlings with healthy new reddish hairy leaves produced at the apex (Fig. 2 B). Such soil was also used for seed germination with excellent results.

The endophytic fungus is normally present in the roots of wild plants and soil surrounding them. Some failures in transplanting *Epigaea repens* from its native habitat may be attributed to removing the plant from its

normal environment containing the endophyte, and the stripping off of epidermis containing the endophyte coils by careless handling (Fig. 1 B). Lack of moisture may well be another cause of failure to establish plants in a new location. A mulch of peat moss or pine needles will help conserve soil moisture (3, p. 94). From these observations it seems fair to conclude that growth of *Epigaea repens* is more vigorous when the roots contain the endophytic fungus, and when the same fungus is abundant in the surrounding soil and humus.

A leaf which rooted at the base of the blade and on the petiole was illustrated in a previous paper (3, Fig. 2 B). Another case of unusual root formation was found in August, 1936, where a leaf fragment buried in peat moss rooted at the base of the blade on the midrib.

#### THE EFFECT OF LIGHT

A pot of 39 recently transplanted seedlings was placed in the insulated greenhouse (2) at the Institute on November 26, 1935, to study the effect of additional light during the winter months. Plants around the outer edge of the pot soon dried out and died, but a circle of ten close to the central water cup survived. During the first month one plant developed more rapidly than the others and started a runner. The seedlings developed elongated red petioles and the new leaves were lifted toward the light. The controls were kept in a shaded greenhouse where growth during the winter months was much slower than in the insulated greenhouse. However, all 36 control plants survived. By late March some of the older leaves in the insulated house were yellowish-green, especially around the edges, and some leaves were wrinkled. By this time the controls had begun vigorous new leaf growth and were growing more rapidly than those in the insulated house. On April 6, 1936, rootlets of both sets showed some cell division and chromosomes. Those in the insulated house had fewer tips showing cell division. Both sets showed fungus coils in the roots, but these were more numerous in the control plants in the shaded house. During the first three months, December to February, growth seemed to be stimulated by the additional light. After that the control plants showed more vigorous growth and better leaf color. The treated plants were returned to the shaded greenhouse on April 6 and within a week showed improved growth. During the season of long daylight *Epigaea* seems to thrive better with at least partial shade.

#### CHROMOSOMES

*Epigaea repens* has a root system characteristic of many of the Ericaceae. While examining the numerous transparent fibrous roots for the presence of the endophytic fungus, it was soon discovered that actively

growing root tips are excellent material for observing living chromosomes (Fig. 2 C). The chromosomes are glistening and of quite different refraction from the rest of the cell contents. There is not much difference in chromosome size in the same cell, but chromosome size seems to vary with cell size (Fig. 2 D). The cells at the meristem just under the root cap are usually seen in side view and it is difficult to be sure of the exact number, which is not exceedingly large. In one root primordia just starting division and seen in the plate view, 14 were counted. The chromosome number needs to be verified by more counts and by pollen mother cells, which have not yet been secured. As arbutus can be grown in a shaded greenhouse the year round, it furnishes excellent material to the teaching botanist for a supply of living chromosomes in root tips. *E. asiatica* (Fig. 2 E) seems to have about the same chromosome number. This may explain the fertile species cross made by Marchant in 1928 (13).

#### SUMMARY

1. The endophytic fungus of *Epigaea repens* L. has been found widely distributed in the stem and roots; on the flowers, pollen, and young ovules; on and in the mature fruit; and on the surface of freshly-ripened seeds. The fungus probably becomes established in young seedlings from the hyphae present on the seed coat.

2. The fungus has been isolated from rooted cuttings, collected plants, flowers, fruits, seeds, and seedlings. The fungus occurs normally in the soil and satisfactory growth of arbutus can be maintained by adding soil containing the endophyte obtained around the roots of vigorous plants. The life history of the mycorrhizal fungus needs further investigation.

3. It has been found that cuttings root readily in several soil mixtures and grow better if the endophytic fungus is present. Seeds can be produced as the result of hand pollination if one has both pistillate and staminate plants. Seeds germinated well when sown soon after ripening; and seedlings develop normally if the mycorrhizal fungus is present. Shade and moisture have been found necessary to promote vigorous growth of trailing arbutus.

4. *Epigaea repens* root tips furnish excellent material for demonstrating living chromosomes.

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# NORMAL-TOBACCO-PLANT PROTEIN AND TOBACCO-MOSAIC-VIRUS PROTEIN AS ANAPHYLACTOGENS AND PRECIPITINOGENS IN THE GUINEA PIG<sup>1,2</sup>

HELEN PURDY BEALE AND BEATRICE CARRIER SEEGAL

## INTRODUCTION

In general, when a guinea pig is injected with an antigen (the anaphylactogen), if a period of ten or more days is allowed to elapse (the period of incubation) a state of sensitivity to the antigen develops in this animal. When the same antigen is injected intravenously into an animal thus sensitized, a severe or fatal shock (anaphylactic shock) usually results. This anaphylactic reaction is so highly specific and can be induced by such small amounts of antigen that it is often utilized for the identification of substances where qualitative chemical methods are difficult or fail.

Once an animal becomes hypersensitive, the anaphylactic state persists unless desensitization is undertaken. A temporary, partial, or absolute desensitization may be accomplished by injecting a series of sublethal doses of the anaphylactogen, usually given subcutaneously or intraperitoneally, which permits a slow absorption and gradual distribution of the antigen. The saturation of tissue antibody by the antigen probably accomplishes this desensitization.

The ability of normal-tobacco, leaf-extract protein and of purified, crystalline, tobacco-mosaic-virus protein to induce anaphylaxis was first investigated in 1936 by Chester (3) who utilized the Schultz-Dale technique. By this *in vitro* method the excised uterine muscle of sensitized animals is suspended in a warmed, oxygenated, Ringer-Locke bath. Upon addition of the specific antigen, contraction of the uterine muscle occurs which is recorded on a kymograph. Chester found normal-tobacco protein highly anaphylactogenic whereas the tobacco-mosaic-virus protein provoked no response. This difference in the antigenic property of normal-tobacco and tobacco-virus protein enabled Chester to demonstrate the presence of common anaphylactogens in chemically purified preparations of normal and virus proteins by a more delicate test than the precipitin reaction. The positive anaphylactic cross reactions obtained by Chester were attributed to non-infective, low-molecular-weight proteins contaminating the purified virus preparations. In 1937 Seastone, Loring, and Ches-

<sup>1</sup> A report on this paper was presented before the 41st General Meeting of the Society of American Bacteriologists, New Haven, Connecticut, December, 1939 (2).

<sup>2</sup> Joint contribution of the Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York, and the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York, New York.

ter (6) confirmed Chester's findings relative to the anaphylactogenic property of normal-tobacco protein and the non-anaphylactogenic property of the virus protein when tested by the Schultz-Dale technique. These authors further demonstrated the anaphylactogenic nature of both normal-tobacco and virus protein when tested in the guinea pig *in vivo*.

#### EXPERIMENTAL PROCEDURE

The experiments reported in this paper deal with a further study of the anaphylactogenic and precipitinogenic properties of normal-tobacco-plant protein and tobacco-mosaic-virus protein when tested in the guinea pig. Some observations on desensitization are included. The anaphylactogenic nature of these proteins was investigated by the method of testing for *in vivo* anaphylactic shock with both homologous and heterologous antigens. The precipitinogenic properties of these proteins in the guinea pig were studied in relation to anaphylaxis and desensitization.

*Preparation of antigens.* Crude, freshly-extracted, tobacco-plant juice is toxic to guinea pigs when injected intravenously. Immediate prostration of a normal guinea pig may result from the injection of as small a dose as 0.4 cc., thereby obscuring the symptom-complex of anaphylaxis in a sensitized animal. The toxic principle is eliminated by dialysis but since the protein content of normal-tobacco juice is low, a concentration of the protein fraction is desirable. Chemical purification not only removes the toxic principle of crude tobacco juice, but permits concentration. Both normal and virus antigens were purified chemically. The normal antigen was prepared by the method described in Seastone, Loring, and Chester's paper (6, p. 409). The plants were frozen, ground, and extracted with disodium phosphate. The juice was then clarified by filtration through Celite and precipitated with saturated ammonium sulphate. The precipitate was removed by filtration through Celite and redissolved in 0.1 M phosphate and reprecipitated. The tobacco-virus protein was prepared by the general procedure outlined by Stanley (7) involving precipitation with 0.4 saturated ammonium sulphate and the alternate adsorption and elution from Celite at different pH levels of approximately 4.5 and 8.00 respectively. A Turkish variety of tobacco (*Nicotiana tabacum* L.) was used for the healthy antigen and for the multiplication of the tobacco-mosaic virus (*Marmor tabaci* var. *vulgare* H.<sup>3</sup>). The purified preparations of both antigens were dialyzed against distilled water or physiological saline (0.85 per cent) and after adding a small piece of thymol were corked and stored in the refrigerator for future use. Before injection micro-Kjeldahl determinations were made and the protein content of each preparation was recorded. The

<sup>3</sup> According to the system of Latin binomials for designating viruses as proposed by Holmes (4).

antigens were diluted so that a test dose never exceeded 2 cc. by volume. The inoculation of susceptible plants showed a high concentration of active virus in the virus antigens and no virus contaminating the normal antigen.

Chester has shown (3) that chemically purified virus protein which has been recrystallized several times still contains inactive, low-molecular-weight protein. Bawden and Pirie demonstrated that this contaminating protein is disintegrated by tryptic digestion without loss of virus infectivity (1). Three tryptic digests of virus protein were also used as antigens. Chemically purified tobacco-virus preparations were made alkaline by the addition of an equal volume of 0.05 M phosphate or boric acid buffer of pH 8.00 to 9.00, commercial trypsin was added in a concentration of 0.002 to 0.005 g. per cc. of virus-buffer mixture, and after final adjustment of the reaction to pH 7.90 to 8.73, digestion was carried out at a temperature of 35° to 37° C. for 44 to 70 hours in the presence of a bacterial antiseptic, toluol or thymol. The tryptic digest was recrystallized several times to remove the trypsin as determined by the inability of the mixture to liquefy alkaline gelatine after incubation at room temperature for 48 hours. The original virus-trypsin mixture produced complete liquefaction of the gelatine when tested under the same conditions. Infectivity tests with recrystallized tryptic digests of virus protein indicated a high concentration of active virus.

The toxicity of the antigens was investigated. Each of four guinea pigs was given an initial intravenous injection of 9.5 mg. of normal-tobacco protein and nine were given intravenous injections of virus protein varying from 12 to 18 mg. and averaging 16 mg. Normal antigen produced no apparent toxic response either upon the initial intravenous injection or during the course of sensitization. The virus antigen induced no symptoms upon intravenous injection other than a drop in temperature ranging from 0.4° to 2.5° F., and in one instance a rise of 2.2° F.

*Sensitization of guinea pigs.* Forty-four guinea pigs weighing from 200 to 300 grams were sensitized by one or more intraperitoneal or intravenous injections of antigen. Sixteen animals were sensitized with normal-tobacco-protein, and twenty-eight with tobacco-virus protein. The animals were tested for anaphylactic shock with either homologous or heterologous antigen. If they survived the test dose those which had been injected with only one of the antigens were retested after another appropriate incubation period. The preinjection temperature was noted and after the injection of a sensitized animal a record of the temperature was kept until recovery was evident. Those animals which died were autopsied. In all cases of death following the test injection of antigen the autopsy findings were typical of anaphylaxis. The constant feature was marked emphysema, the lungs filling the entire thoracic cavity. Small hemorrhages in the lungs and



heart muscle were usually present also. However, death could be ascribed to asphyxiation following constriction of the bronchioles and the trapping of air in the distended alveoli.

In an effort to determine the optimal conditions for fatal shock, a wide range of sensitizing and test doses and varying periods of incubation were used. By subjecting the animals to great variation in experimental conditions, information was obtained which made it possible to induce fatal shock with regularity when virus protein was used as anaphylactogen. An optimal set of conditions for inducing fatal shock with normal-tobacco protein has not been determined.

Fifteen separate attempts to induce shock with normal-tobacco protein after sensitization with 0.3 to 4.0 mg. of the homologous antigen resulted in complete failure and six attempts to shock with virus protein after sensitization with from 2.5 to 4.0 mg. of antigen also resulted in no reaction so that all tests on animals sensitized with less than 4.5 mg. of either antigen have been omitted from the tables.

The optimal incubation period proved to be 21 days or longer. The optimal shocking dose varied with the size of the sensitizing dose and the antibody content of the serum as will become apparent following the presentation of the data.

*Results of anaphylactic tests with normal and virus protein.* In Table I the results of the experiments in anaphylaxis with the homologous antigen are grouped according to the response of the animal to the treatment given. In every case the range of sensitizing dose, test dose, and period of incubation are given as well as the average for the group. The results indicate that both normal-tobacco-plant protein and tobacco-mosaic-virus protein are capable of inducing severe or fatal anaphylactic shock. More regular results, however, were obtained with the virus protein.

A three dimensional graph (Fig. 1) was constructed to facilitate the examination of the data. The vertical axis was used to indicate the mg. protein injected for sensitization of the animal, while the two lateral axes, the abscissa and ordinate, were assigned respectively to the number of days comprising the period of incubation and the mg. protein used as a test dose. The dark-capped perpendicular rods represent the animals sensitized with tobacco-virus protein; the light-capped rods are used to designate guinea pigs sensitized with normal-tobacco protein. The reaction of the sensitized animal to the injected test dose is indicated on the cap at the top of the vertical rod as 0 or no reaction; + or mild shock; ++ or moderate shock; +++ or severe shock; F or fatal shock. The details of these reactions are given in the footnote in Table I. When two or more treatments were similar or identical, the average treatment is represented by a single rod and the individual reactions to the several test doses are recorded on the cap. The height of the vertical rod is an indication of the



total number of mg. protein used for sensitization, which in some instances represents a single dose and in other cases several doses, injected after varying periods of incubation until the guinea pig either succumbed to fatal anaphylaxis (F), died of other causes, or was discarded. Since the same antigenic unit (mg. protein) was used for both sensitizing and test doses, the two are drawn to the same scale on the graph and can be directly

TABLE I  
ANAPHYLAXIS TO NORMAL AND VIRUS PROTEIN

No. of		Antigen	Sensitizing dose, mg. protein		Test dose, mg. protein		Incubation period, days		Results	
Guinea pigs	Tests		Range	Av.	Range	Av.	Range	Av.	No. of guinea pigs	Reaction*
8	16	Normal-tobacco protein	38 - 45	42	9.5-13	12	22-46	30	3	F
			19 - 48	37	6.5-12	10	15-46	33	4	+
			19 - 44	24	6 - 16	10	22-46	35	9	o
27	44	Tobacco-virus protein	29 - 75	45	4 - 18	12	27-59	36	14	F
			34 - 74	44	4.5-20	11	28-43	38	5	+++
			4.5 - 85	29	3.5-18	12	5-46	24	9	++
			8 - 69	40	5.5-19	16	5-40	18	7	+
			4.5-103	27	0.5-18	7.5	8-39	18	9	o

\* o=no reaction; +=mild shock, sneezing, scratching of nose, defecation, urination, average drop in temp. 2.3° F.; ++=moderate shock, guinea pig prostrate, recovery slow, average drop in temp. 3.8° F.; +++=severe shock, cyanosis, symptoms similar to those of fatal anaphylaxis, average drop in temp. >6.2° F.; F=fatal shock with postmortem findings characteristic of anaphylaxis.

compared. Caps indicating single reactions were also used so that the conditions under which a given reaction occurred could be examined critically.

An examination of the graph (Fig. 1) will disclose the wide range of experimental conditions to which the guinea pigs were subjected. Eight animals sensitized to normal-tobacco protein were subjected to a total of 16 test doses of the homologous antigen; 27 guinea pigs sensitized to tobacco-virus protein were given 50 test doses of virus antigen. A fair basis for comparison of the two antigens is obtained by considering only those tests which conform to a minimal set of experimental conditions capable of inducing *fatal* anaphylaxis with either of the antigens. An inspection of the various treatments and the resultant reaction discloses that these minimal requirements are 4 mg. protein for the test dose, 29 mg. for sensitization, and 22 days for the shortest period of incubation under the conditions of these experiments. Seven tests on as many guinea pigs sensitized and subsequently tested with normal-tobacco antigen conformed to these minimal test conditions. Two of the animals failed to react, two gave a mild response, and three succumbed to fatal anaphylaxis. Twenty-eight tests on 22 animals sensitized and tested with tobacco-virus protein satis-

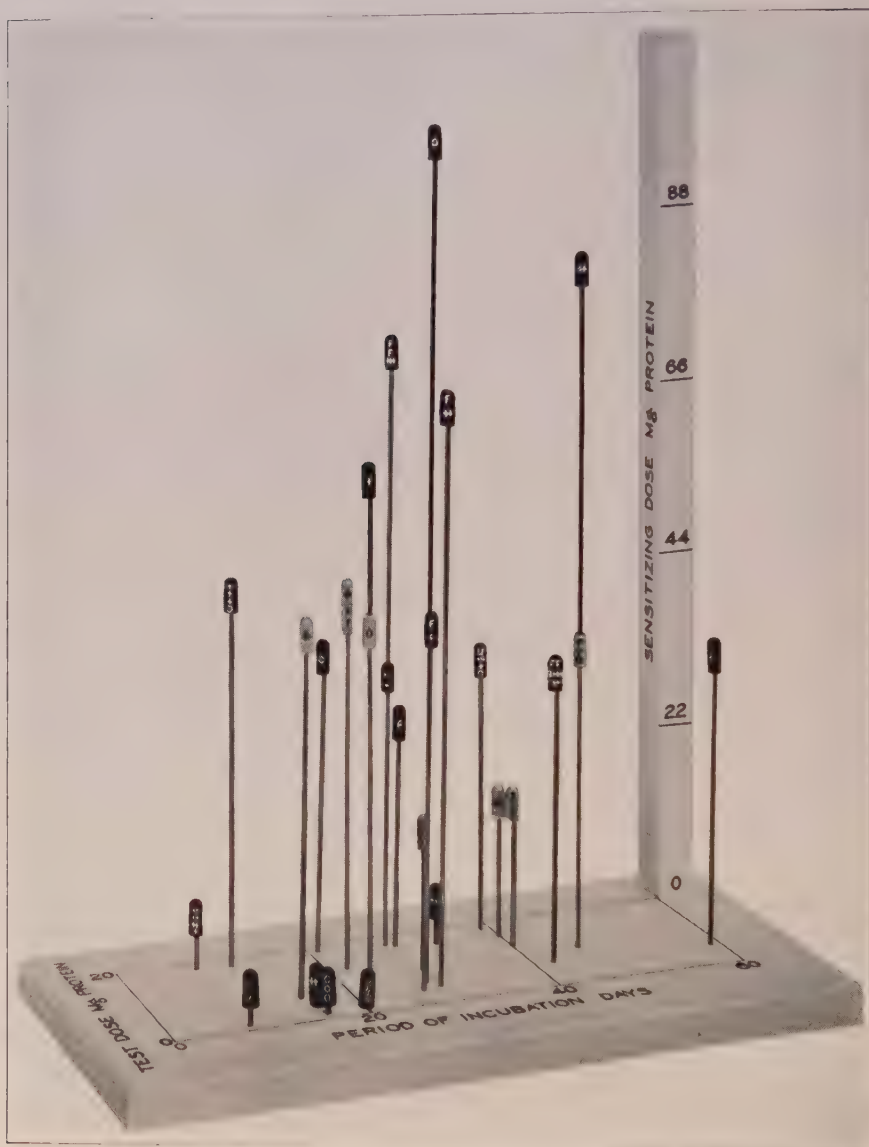


FIGURE 1. Graph showing response to various treatments used in sensitizing guinea pigs with normal-tobacco protein and tobacco-virus protein. Light-capped rods represent normal protein and dark-capped rods, virus protein. o=no reaction, +=mild, ++=moderate, +++=severe, and F=fatal shock. The sensitizing dose (mg. protein) is recorded on the vertical axis, the period of incubation (days) on the abscissa, the test dose (mg. protein) on the ordinate.

fied the minimal requirements for inclusion in this group of experiments. Two of the tests were negative, three resulted in mild reactions, four in moderate, five in severe, while fourteen of the tests resulted in fatal shock.

The conditions under which fatal anaphylaxis was induced most frequently with tobacco-mosaic-virus protein were a single sensitizing dose of 37 mg. followed by a test dose in about 40 days of 18 mg. protein. Four guinea pigs receiving this treatment succumbed to fatal anaphylaxis in from 3 to 5 minutes. After sensitization with 37 mg. virus protein, fatal shock was induced in three cases and severe shock in two instances with test doses ranging from 4.5 to 11 mg. virus protein. In a single instance, an animal was tested for sensitivity to the virus protein 59 days after a single sensitizing dose of 37 mg. of the homologous antigen and succumbed to a test dose of 5.5 mg. within 5 minutes after injection.

Shock was induced with tryptic digests of tobacco-mosaic-virus protein following sensitization with either non-tryptic or tryptic digests of virus protein. No reaction was obtained in a normal guinea pig injected with 1 cc. of a 50,000 saline (0.85 per cent) dilution of commercial trypsin equal in concentration to the trace of active enzyme present in the tryptic digest of virus antigen, as determined by the ability of such a chemically purified digest to liquefy alkaline gelatine.

In the two instances attempted, fatal anaphylaxis occurred when guinea pigs were sensitized with chemically purified virus protein and shocked with intravenous injections of 2 cc. test doses of filtered, dialyzed juice from a tobacco plant affected with mosaic disease. A normal guinea pig injected intravenously with a dose of tobacco-virus juice equivalent to that which induced fatal anaphylaxis in the two sensitized guinea pigs gave no evidence of any reaction to the injection, thereby providing evidence of the non-toxicity of the tobacco-virus juice.

*Cross reactions.* In Table II the results of cross tests are recorded. Six guinea pigs sensitized with doses of normal-tobacco antigen ranging from 38 to 60 mg. protein were subsequently tested with chemically purified but non-tryptic digested tobacco-virus protein in doses ranging from 15 to 31 mg. after intervals between the last sensitizing dose and test dose varying from 19 to 96 days. Such treatment provoked no response in three instances, mild response in two cases, and a severe reaction barely escaping death in a single case. Three separate preparations of virus antigen were used in these cross tests. Four guinea pigs were sensitized with from 19 to 43 mg. virus protein and tested with doses of normal-tobacco protein amounting to 12 to 14 mg. after 13- to 40-day periods of incubation. The result was negative in three cases and in the fourth a severe reaction was provoked. Two and one-half hours later two of these animals were injected with tobacco-mosaic-virus antigen. Guinea pig No. 623 suffered fatal anaphylactic shock and No. 629 a very severe or +++ reaction, thus

demonstrating that the normal-plant antigen had failed to desensitize the animals to the virus antigen.

*Desensitization.* In several cases it was noted that an animal which had survived a severe reaction to virus protein, the homologous antigen, was not entirely desensitized because it succumbed to an injection of the same antigen when administered intravenously on the following day. This ob-

TABLE II  
ANAPHYLACTOGENS IN COMMON IN NORMAL AND TOBACCO-VIRUS PROTEIN

Guinea pig No.	Sensitizing dose, mg. protein	Test dose, mg. protein	Incubation period, days	Result*
558	38 Normal	18 Virus	96	+++
557	57 "	15 "	48	o
160	60 "	29 "	19	+
615	51 "	31 "	19	o
158	51 "	31 "	19	o
559	57 "	18 "	49	+
629	37 Virus	12 Normal	40	o
623	43 "	12 "	27	+++
526	19 "	13 "	28	o
536	29 "	14 "	13	o

\* See footnote in Table I.

servation led to a more detailed study of this second-day response. Twenty guinea pigs which were sensitized to virus protein and exhibited o to +++ reactions to a test dose were given a second test dose of the same antigen on the following day. In three instances an interval of 2 days was allowed to elapse before a second test dose was given.

*Results of desensitization tests.* The results of these tests are presented in detail in Table III. Eight out of 20 guinea pigs sensitized with virus protein succumbed to the injection administered on the day succeeding the first test dose; 2 of the animals reacted severely to the second-day injection in response to three separate tests; 2 of them gave moderate responses to three tests; 1 exhibited mild shock; and 7 failed entirely to respond. The animals succumbing to shock gave the symptom-picture and postmortem findings characteristic of anaphylactic shock. The time between the injection and death was generally prolonged with this group of guinea pigs and was as follows for the 8 animals: 4, 7, 8, 10, 20, and 21 minutes, 1 hour 28 minutes, and 2 hours 15 minutes.

To determine the possible toxic effect of such large doses of antigen when injected into the animals at short intervals, 12 normal guinea pigs were given two successive doses of tobacco-virus protein averaging 15 and 16 mg. respectively, at an average interval of 26 hours, and in no case was a response provoked by either the first or the second injection.



Thirteen guinea pigs sensitized to normal-tobacco protein averaging 17 mg. were given test doses on two successive days amounting to an average of 12 and 11 mg. respectively. The average interval between the two injections was 24 hours. As indicated in Table III, the response to the first

TABLE III  
TESTING FOR DESENSITIZATION WITH THE HOMOLOGOUS ANTIGEN

No. of		Average dosage, mg. protein		Average incubation period		Results of tests*		
Guinea pigs	Tests	Sensitizing dose	Test dose		No. days since last sensitizing dose	No. hrs. between tests 1 and 2	1	2
			1	2				
8	8	44 Virus	14.0	14.5	23	23.5	+++, 4 ++, 2 +, o	F
2	3	19 "	17	16	24	25.5	2 ++, +	+++
2	3	69 "	14	14	18	23.5	2 +, o	++
1	1	29 "	18	17	42	23.5	++	+
7	7	35 "	17	16	29	24	2 +++, 3 +, 2 o	o
Guinea pig No.								
561		27 Virus	17	17	27	46.5	+	+
622		37 "	4.5	4.5	41	47.5	+++	++
562		34 "	17	17	27	47	++	o
No. of								
Guinea pigs	Tests							
13	16	17 Normal	12	11	33	24	++, 2+, 13 o	o
12	Not sensitized, tested with virus protein		15	16	o	26	o	o
2	Not sensitized, tested with normal protein		9.5	8	o	29	o	o

\* See footnote in Table I.

injection varied from o to ++, but in none of the 16 tests to which these 13 animals were subjected was there any evidence of shock following the second-day injection. As a control on normal-tobacco protein used as an antigen for injection on two successive days, two normal guinea pigs were given a first injection of 9.5 mg. protein each and approximately 29 hours later they were injected with an average of 8 mg. more of the antigen. No reaction resulted.

## SERUM REACTIONS

*Precipitin and neutralization reactions.* In an effort to explain the failure to desensitize animals with one injection of the homologous antigen, tobacco-virus protein, it was decided to investigate the relative precipitin titer of the serum from sensitized animals both before and after injection of a test dose.

Seven serums from guinea pigs sensitized to normal-tobacco protein were tested for precipitin to either homologous antigen or virus antigen with entirely negative results. In the rabbit also the normal protein is a poor antigen (5).

Thirteen separate antiviral serums, including several samples of serum from the same animal drawn at different times, all contained precipitin to virus protein.

An attempt was made to determine what possible relation the precipitin titer of a given animal, sensitized to virus protein, might have to the type of response elicited by the test dose. This phase of the work is not completed but enough information has been obtained to warrant inclusion of the results thus far in this article. A small sample of blood was drawn from the heart of an animal just previous to injection of the test dose and in some cases after injection. If the animal succumbed to anaphylaxis, a sample of blood was drawn during postmortem examination. If the guinea pig survived and received a second injection on the following day, the procedure of drawing the samples of blood was repeated. By this means it was possible to examine the titer of the antiserum for a rise or fall of precipitins following injection of antigen.

As a rule, the precipitate formed in virus-antiserum mixtures was so heavy that it was impossible to compare the titer of two antisera without first reducing their precipitin content by partial absorption. The usual precipitin-absorption technique was employed in comparing the relative titers of the samples of antisera. Virus antigen was added to the antisera in known amounts, then thoroughly mixed and allowed to stand for one hour in a water bath at  $37.5^{\circ}\text{C}$ . after which the tubes were placed in the refrigerator overnight. The following day the presence or absence of precipitate was recorded, the tubes centrifuged, and the supernatants drawn off. These supernatants were divided into two portions, the one tested for the presence of antigen (virus), with a high-titered rabbit antiviral serum, the other tested for the further presence of precipitin by the addition of more virus antigen. In no case had an excess of virus been used for absorption since none of the supernatant fluids (absorbed antisera) precipitated upon addition of the rabbit antiviral serum. Thus, after partial absorption of two or more samples of antiserum with an equal volume of the same antigen preparation, the remaining precipitin content was

determined by further titration and served as an indication of the relative titer of the original unabsorbed antisera.

In a single experiment a comparison was made of the ability of two samples of antiviral serum of different precipitin titer to neutralize an equal volume of virus protein. The antiserum-virus mixtures in this experiment were thoroughly shaken and inoculated on opposite halves of leaves of the susceptible *Nicotiana glutinosa* L. plant.

*Results of serum-precipitin titrations in shocked, virus-sensitized animals.* Guinea pigs No. 630 and No. 154 of Table IV were bled before the shocking

TABLE IV  
COMPARISON OF PRECIPITIN TITER OF ANTIVIRUS SERUMS AFTER PARTIAL ABSORPTION

Antiserum No.	Antiserum taken	Dilution of absorbed antiserum	Precipitate formed upon addition of virus**
630	P.I.*	1 in 10 1 in 50	+++ +
	P.A.	1 in 10 1 in 50	+ ±
154	P.I.	Undiluted†	+
	P.A.	"	0
629	P.I. on Nov. 16	Undiluted†	+
	F.I.	"	±
	P.I. on Nov. 17	"	+
	P.A.	"	0

\* P.I. = preinjection; F.I. = following injection; P.A. = postanaphylactic death.

\*\* 0 = no precipitate; ± = slight precipitate; + = moderate precipitate; ++ = heavy precipitate; +++ = very heavy precipitate; ++++ = precipitate so heavy it may fill from  $\frac{1}{2}$  to  $\frac{3}{4}$  tube.

† When mixed with rabbit antiviral serum, this absorbed guinea pig antiserum gave no precipitate which indicates the absence of excess virus.

injection of antigen and blood was obtained again following the anaphylactic death of the animals. The striking finding in these two animals, as presented in the table, was that the shocking injection of antigen reduced but did not eliminate the circulating precipitin. Guinea pig No. 629, also described in Table IV, was of even greater interest in interpreting the failure to desensitize with a single shocking dose of antigen. The precipitin content of the circulating blood was reduced after a non-fatal dose of antigen. The following day there was evidence of a recovery in precipitin titer. At this time another injection of antigen produced fatal shock but again failed to remove all the circulating antibodies.

The reduction of circulating precipitin is demonstrated in the case of guinea pig No. 623 in Table V by employing the method of testing for the

neutralization of infective power of virus. This method is probably a more nearly quantitative method than the method of absorption previously employed. The serum drawn before the test injection of antigen was able to neutralize virus completely when diluted 1:8 and was quite effective

TABLE V  
COMPARISON OF PRECIPITIN TITER OF VARIOUS SAMPLES OF ANTIVIRUS SERUM AND  
THEIR ABILITY TO INACTIVATE VIRUS

Antiserum No.	Antiserum drawn*	Dilution of antiserum	Precipitate after addition of virus**	No. viroous lesions on plant
623	P.I.	1 in 2	++++	0
	P.A.	"	++	0
	P.I.	1 in 4	+++	1
	P.A.	"	+	4
	P.I.	1 in 8	++	1
	P.A.	"	0	30
	P.I.	1 in 16	0	10
	P.A.	"	0	85
	Virus	Saline		77

\* See footnote \* in Table IV.

\*\* See footnote \*\* in Table IV.

even when diluted 1:16. The serum after fatal shock was relatively poor at dilution 1:8 and was completely lacking in neutralizing power when diluted 1:16. This experiment again demonstrated the reduction of precipitin following the shocking injection of antigen but nevertheless shows their persistence in very appreciable quantities.

#### DISCUSSION

Both normal-tobacco-plant protein and tobacco-mosaic-virus protein as prepared in these experiments have proved less anaphylactogenic in the guinea pig than the animal proteins usually employed. Less than 1 mg. of horse serum protein is adequate to sensitize the guinea pig so acutely that 1 to 2 mg. of the same protein will induce fatal shock, whereas, 4 mg. of either normal or virus protein proved insufficient to induce critical sensitization. The smallest amount of normal-plant protein which induced fatal shock was 9.5 mg. and of tobacco-virus protein 4 mg.

These amounts of protein are considerably greater than those reported by Chester sufficient to produce anaphylaxis. An explanation for this is not apparent, but may possibly lie in differences inherent in various preparations of antigen. The experiments described above certainly discourage the use of the *in vivo* anaphylactic method for identifying traces of contaminating normal-tobacco-plant protein in virus preparations since in our hands this protein has not proved highly anaphylactogenic.



Serums from guinea pigs injected with normal-tobacco-plant protein did not prove to have any demonstrable precipitin, whereas serums from animals injected with virus protein showed high precipitin titers. Chester is of the opinion that since two animals may have the same precipitin content in their serums and still one may be sensitive and the other insensitive to a test dose of the homologous antigen, the indication is that the production of precipitin develops independently of the anaphylactic state (3). Our results are suggestive of the possibility that the precipitin titer of a sensitized guinea pig is an important factor in determining whether the animal will survive or succumb to a test dose of the homologous antigen. The circulating antibody may be able to prevent antigen from reaching the tissue antibody and thereby afford protection. Or it is possible that the phenomenon of inhibition in the presence of excess antibody or antigen enters into the *in vivo* reaction. It is hoped that more quantitative experimentation along these lines may be undertaken.

#### SUMMARY AND CONCLUSIONS

1. An optimal set of conditions was obtained by which guinea pigs could be severely or fatally shocked with tobacco-mosaic-virus protein. These conditions were: a single sensitizing dose of 37 mg. virus protein followed by a test dose in about 40 days of 18 mg. of the same protein.
2. No such uniform results were obtained with normal-tobacco-plant protein.
3. Because normal-tobacco-plant protein has failed to shock consistently when employed in small quantities, the *in vivo* method of anaphylaxis seems unsuited for detecting traces of normal-tobacco protein which may contaminate chemically purified preparations of virus protein.
4. Desensitization of guinea pigs sensitized to normal-tobacco protein occurred following a non-fatal shock.
5. Desensitization of guinea pigs sensitized to tobacco-virus protein often failed to occur following a non-fatal shock.
6. Precipitin to normal-tobacco-plant protein was not demonstrable in the serum of guinea pigs injected with this antigen.
7. Precipitin to tobacco-mosaic-virus protein was regularly demonstrable in the serum of animals injected with this antigen.
8. The failure of desensitization with virus protein is believed to be due to the high precipitin content of the guinea pig serum. Amounts of virus protein adequate to induce severe or even fatal shock have so far proved insufficient to remove all of the circulating antibody. Under such conditions, surviving animals were frequently susceptible to severe or fatal shock the following day.
9. This difficulty in desensitization associated with the persistence of circulating antibody suggests a correlation between anaphylaxis and the precipitin antibody.

10. The neutralizing antibody to tobacco-virus antigen in guinea pig serum decreases with the precipitin titer.

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## FURTHER STUDIES ON THE RAPID DETERMINATION OF THE GERMINATIVE CAPACITY OF SEEDS

FLORENCE FLEMION

Many seeds require from four to six months for germination to occur. A rapid method of determining the germinative capacity of such seeds is especially important. Results (6) have shown that the observation of the behavior of excised embryos serves as a good test for the viability of these seeds. No matter how dormant a seed may be, whenever the excised embryo is placed on moist filter paper at room temperature, some indication of its viability is obtained within ten days. The non-viable embryos deteriorate while the viable embryos either remain intact or show various types of hypocotyl development and cotyledon enlargement. The percentage of viable embryos thus obtained is comparable with the percentage obtained when the intact seeds have been subjected to the optimum conditions for germination. While the time required to test the viability of these seeds is greatly reduced, this rapid method requires considerable care for the excision of the embryos. Any difficulty that may arise with this test is in the removal of the embryo from the outer and inner seed coats.

Several methods of excising embryos were described earlier (5, 6, 7), but these are not applicable to all types of seeds. Certain pretreatments have been developed which greatly facilitate the excision of the embryos. The pretreatments vary with different types of seeds, for some readily imbibe water, while some require the use of various devices to remove the outer coats in order to imbibe water. The various techniques used for excising the embryos of 33 species representing 11 families are discussed below. Many kinds of seeds are included, ranging from non-dormant seeds to some of the hard-coated seeds with dormant immature embryos.

### MATERIALS AND METHODS

The seeds were collected in the vicinity of the Institute at Yonkers, New York, or obtained from either commercial seedsmen or private collectors.

The hard outer coats of some seeds were removed with the aid of a cracking device (5), and the coats of others were treated with concentrated sulphuric acid prior to the cracking. Regardless of method, after the outer coats were off the seeds were soaked in water overnight and the inner coats removed by making a slit with a sharp instrument. Sometimes the intact seeds were mixed in moist peat moss for a short period either with or without a previous acid treatment. In some cases, this facilitated the cracking of the outer coats, while in other cases, due to adequate imbibition of

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water, it either simplified the excision or accelerated the subsequent development of the embryos. Sometimes soaking the intact seed in water overnight was adequate, for a slit could then be easily made through both coats with a sharp scalpel. When the endosperm was very thick several slits were made and after an additional period in water the embryo could be removed.

When the seeds were treated with concentrated sulphuric acid, a quantity of acid equal to about five times the volume of the seeds was used. The mixture was stirred frequently. At the end of the treatment the acid was removed, the seeds thoroughly washed in running tap water, and then kept in a large volume of water for at least an hour to remove any remaining acid. Certain freshly-harvested seeds were not treated with acid until after several weeks of drying at room temperature, because with fresh seeds the acid penetrated and destroyed the embryos due to the higher moisture content of the seeds.

As the embryos were excised they were placed in water and later transferred to moist filter paper in a Petri dish (10 pieces of 15 cm. paper and 30 cc. of tap water) at room temperature. During the warm summer months when the temperature was above 24° C., the dishes were placed in a 20° C. electrically-controlled oven kept in a refrigerator room. When pine embryos were used, only 20 to 25 cc. of water were added to the filter paper as they develop best under this moisture condition.

In each case the value for the germinative capacity of the various lots of seeds studied was compared with the actual per cent germination in a regular germination test. The percentage viable is sometimes higher by the excision method. This may be due in some cases to lack of optimum conditions for after-ripening and subsequent germination. Duplicate lots of 50 or 100 seeds were used for the germination tests, while duplicate lots of 30 or 50 seeds were used for the excision tests. Since embryos only were used for the Petri dish method, proper corrections were made when some of the seeds were empty or contained shriveled embryos. The concordance between the results of the rapid method and the actual germination obtained is shown in the various tables.

## RESULTS

### SEEDS REQUIRING PREVIOUS ACID TREATMENTS

Five of the ten species studied which have hard coats had to be treated with concentrated sulphuric acid before the outer coats could be removed. After subsequent imbibition of water the embryos could be excised.

*Red bud.* After treating with acid for 45 minutes or filing, the hard coats of *Cercis canadensis* L. readily imbibe water. Following either treatment the embryos are easily excised after soaking in water overnight at room temperature. The large yellow embryos develop rapidly when viable. For



TABLE I

RESULTS OBTAINED WITH EXCISED EMBRYOS AND ACTUAL GERMINATION TESTS  
IN TESTING THE GERMINATIVE CAPACITY OF VARIOUS SEEDS

Family	Species	Crop	Experiment started, date	Excision method		Germination method	
				% viable	Time, days	%	Time, days
Bignoniaceae	<i>Campsis radicans</i>	1934 1939	Nov. 1939 " "	0 6	10 6	0 3	57 57
	<i>Catalpa speciosa</i> Warder	1936	" "	50*	3-5	13	51
		1939	" "	100	"	96	28
		1937	June 1940	84	"	78	47
		1938	" "	92	"	66	42
		1939	" "	96	"	92	16
Elaeagnaceae	<i>Shepherdia argentea</i>	?	Jan. 1939	85	5	88	41
Leguminosae	<i>Cercis canadensis</i>	1936	Oct. 1939	94	6	77	48
		1939	" "	93	6	78	107
Oleaceae	<i>Chionanthus retusa</i>	1938	Nov. 1939	97	21	87	213
	" <i>virginica</i>	"	" "	87	"	81	"
		"	" "	91	"	86	"
		"	" "	95	"	88	"
	<i>Menodora scabra</i>	?	May 1939	96	2	90	7
	" <i>scoparia</i>	?	" "	80	6	67	14
Pinaceae	<i>Pinus banksiana</i> Lamb.	1938	Jan. 1939	90	12	92	43
	" <i>echinata</i> Mill.	?	Jan. 1940	82	10	50	60
	" "	?	" "	87	10	77	60
	" <i>excelsa</i> Wall.	?	April "	71	10	53	41
	" <i>nigra</i> Arnold (P. <i>laricio</i> Poir.)	?	" "	74	9	68	44
	" <i>resinosa</i> Ait.	?	Jan. "	65	10	58	91
	" <i>rigida</i> Mill.	1938	" "	94	12	100	61
	" <i>strobus</i> L.	?	" "	92	10	66	36
	" <i>thunbergii</i> Parl. (P. <i>massoniana</i> Sieb. & Zucc.)	?	" "	73	12	79	83
	" <i>virginiana</i> Mill.	?	April "	82	12	76	53
	<i>Pseudotsuga taxifolia</i> Brit.	?	June 1940	49	10	47	56
Rosaceae	var. <i>viridens</i> Schneid.	1935	" "	2	"	0	"
		1939	" "	65	"	43	"
	<i>Crataegus crus-galli</i>	1935	June 1939	0	15	0	350
		1937	" "	32	15	27	"
	<i>Sorbus aucuparia</i>	1931	Sept. 1938	0	11	1	270
		1936	" "	89	"	79	"
		1938	" "	100	"	99	"
Rosaceae	<i>Pyrus ussuriensis</i>	1938	Oct. 1939	64	7	50	167
	Chokecherry	1938	Sept. 1939	97	21	93	250
	Mahaleb cherry	1939	Mar. 1940	91	5-7	64	182
	Myrobalan plum	1937	June 1938	32	5-7	38	67
	Wild plum	1939	Oct. 1939	97	14	85	318
Verbenaceae	<i>Callicarpa purpurea</i>	1938	May 1939	78	7	78	118

\* Sluggish development, seeds low in vigor.

the germination tests (Table I) the seeds were treated with acid for 45 minutes and planted after being kept in moist peat for various periods at 5° C.

*Callicarpa purpurea* Juss. The seeds were treated with acid for 7 minutes, then soaked in water. When excising, the two halves of the outer coat could be separated by using the tip of the scalpel and the inner coats removed by slitting with the scalpel. In the test shown in Table I, 78 per cent were viable (within 7 days) in the Petri dish test, and 78 per cent germinated when intact seeds were planted after three months in moist peat at 5° C.

*Buffalo berry*. The seeds of *Shepherdia argentea* Nutt. were treated with acid for 40 minutes. While subsequently imbibing water many of the outer coats split apart. By the fifth day the viable embryos had either developed into seedlings or had green expanded cotyledons, while the non-viable embryos had deteriorated. Seeds treated with acid for 40 minutes, then kept in moist peat moss for 40 days at 15° to 30° C. alternated daily, produced 88 per cent germination, while by the excision method the germinative capacity was found to be 85 per cent (Table I).

*Snowberry*. The embryos of *Symphoricarpos racemosus* Michx. are very difficult to excise. After the seeds were treated with acid and allowed to remain for a short period in moist peat moss, not only was the excising much easier, but the subsequent development of the embryos was more rapid. Within 10 days on moist filter paper the viable embryos either remained intact or developed a slight greenish tinge, while some elongated. To differentiate between intact, firm, viable embryos and the non-viable which are soft, but have not yet either molded or discolored, the embryos are tapped with a needle. Since the embryos were quite small a low-powered binocular was used.

An experiment was designed to determine the effect of various temperature pretreatments on the percentage viable obtained when subsequently excised. Five different crops and nine variations of temperature were used, while the acid treatment was constant. The results are shown in Table II. The type of temperature pretreatment within a crop did not significantly affect the germinative capacity obtained. However, when seeds treated with acid for 45 minutes were mixed in moist peat moss and kept at 5° C. for several weeks, not only did the endosperm imbibe water, but the immature embryos developed sufficiently so that excising is much easier. Also, some of the non-viable embryos disintegrated during this period.

This is the most difficult seed found so far to excise, for not only are the embryos dormant and immature, but the coats are unusually tough. Under optimum conditions germination can be obtained within four to six months. By the excision method the viability of these seeds can be determined within six weeks.

*Hawthorn*. Many embryos are injured when the intact seeds are cracked. When the seeds are cracked following an acid treatment and a subsequent soaking overnight, the carpels split apart at the dehiscent layer with very little injury to the embryos. An acid treatment of 165 minutes was found to be the best for *Crataegus crus-galli* L. The inner coats are removed by

TABLE II

EFFECT OF VARIOUS PRETREATMENTS UPON SUBSEQUENT VIABILITY MEASUREMENTS OBTAINED WHEN THE EXCISION METHOD IS USED FOR DETERMINING THE VIABILITY OF SYMPHORICARPOS RACEMOSUS SEEDS\*

Crop	Germination method	Per cent viable								
		Excision method**								
		Weeks at 5° C.					Weeks at 25° C.		2 wks. at 25° C. followed by 1 wk. at 5° C.	2 wks. at 25° C. followed by 2 wks. at 5° C.
		0	2	3	4	5	2	4		
1937	66	81	97	82	79	94	83	79	83	93
1936	71	72	69	80	81	90	66	89	75	80
1934	66	64	78	77	62	79	88	65	77	87
1933	50	70	59	62	70	67	71	79	64	72
1927	0	—	8	0	0	0	10	0	0	0

\* Lots of 50 seeds treated at start of experiment in April 1938 with concentrated sulphuric acid for 45 minutes.

\*\* Seeds kept in moist peat moss prior to excision.

slitting with a sharp scalpel. The percentage viable can be determined by the seventh day after the excised embryos are placed on moist filter paper. The results with two crops are shown in Table I. The germinations were obtained by mixing acid-treated seeds in moist peat and keeping at 5° C. for 11 months.

#### SEEDS REQUIRING THE CRACKING OF HARD OUTER COATS

For five species of hard-coated seeds the acid pretreatments were not necessary, but instead the outer coats were removed by using the cracking machine. Some were kept in moist peat moss prior to removing the outer coats while others were kept in peat after the cracking.

*Mahaleb cherry and myrobolan plum*. After removal of the outer coats of *Prunus mahaleb* L. and *P. cerasifera* Ehrh. the inner coats were removed when subsequently soaked overnight in water. After five to seven days on moist filter paper 91 and 32 per cent respectively were viable (Table I), while the germinations obtained by mixing the intact seeds in moist peat and keeping at 5° C. were 64 and 38 per cent.

*Wild plum and chokecherry*. Many of the embryos of *Prunus americana* Marsh. and of *P. virginiana* L. are injured when the outer coats are cracked.

But when the intact seeds are kept in moist peat for a short period (one week for plum and two weeks for chokecherry) at room temperature, very few are injured when cracked, for the coats split apart readily along the dehiscent layer. In addition, the embryos can be excised immediately after cracking as sufficient water has been imbibed. By the seventh day on moist filter paper the non-viable embryos have deteriorated. The results in Tables I and III compare the Petri dish method with the germinations obtained

TABLE III

EFFECT OF KEEPING SEEDS IN MOIST PEAT MOSS AT VARIOUS TEMPERATURES PRIOR TO EXCISION UPON THE SUBSEQUENT RESULTS OBTAINED BY THE RAPID VIABILITY TEST

Species	Crop	Exp. started, date	Per cent viable							
			Germination method	Excision method						
				Weeks at 25° C.				Weeks at 5° C.		
				0	1	2	3	1	2	3
Ben Davis apple	1938	July 1939	96	100	96	100	—	93	100	—
McIntosh apple	"	" "	86	89	90	89	—	88	90	—
Northern Spy apple	"	" "	97	85	94	100	—	98	96	—
Northwest Greening apple	"	" "	76	61	88	86	—	90	92	—
Chokecherry	1938	Nov. 1938	95	40	97	99	92	83	93	98
Japanese quince	1937	April 1938	97	86	100	100	96	88	98	91

when the intact seeds were mixed in moist peat and kept at 5° C. The percentage of viable embryos obtained when intact chokecherry seeds were pretreated in moist peat for various periods is shown in Table III. The low percentage obtained in the control (kept 0 weeks in moist peat) is due to injury while cracking. The absence of injury and ease in excising when held in peat is not indicated in the tables.

*Fringe tree.* These seeds have a thin outer shell which must be removed by cracking and then the seeds are mixed in peat and kept at 20° C. for at least five days. Seeds not given this pretreatment do not imbibe sufficient water, and as a result many embryos are injured during excision. Due to this injury brown areas appear during the subsequent development of the embryos on moist filter paper. The results obtained with *Chionanthus retusa* Lindl. (*C. chinensis* Maxim.) and *C. virginica* L. seeds kept in moist peat for two weeks at 20° C. prior to excising are shown in Table I. Later it was found that five days were adequate for this pretreatment. For germination the seeds (outer shells removed) were mixed in moist peat and held at 20° C. for 7 months. In *Chionanthus* the roots are produced at 20° C., but the shoots do not appear until after a period at low temperature. For the germination tests only the root production was used.



## SEEDS RESPONDING TO A SHORT PERIOD IN MOIST PEAT MOSS

For some species it was found desirable to subject intact seeds for five to ten days in moist peat moss before excising the embryos.

*Japanese quince.* When intact seeds of *Chaenomeles japonica* Lindl. are kept in moist peat for a week or two, the subsequent development of the embryos is much speedier than when the embryos are excised after soaking in water overnight. The results in Table III are not presented in a form to show this accelerated development, but they do show the close agreement of the two methods.

*Pine and Douglas fir.* Since the earlier publication (6) it has been found that there are several advantages to mixing the intact seeds in moist peat and keeping at room temperature for five days. After such pretreatment the excising is speedy and very few of these soft embryos are injured, due to the imbibition of water. Frequently, a considerable number in a given sample need not be excised, for some may have germinated, while some of the non-viable seeds have disintegrated. Also, the subsequent development of the remaining seeds that must be excised is very rapid. The percentage viable is determined on the tenth day; that is, five days in peat followed by five days in the Petri dishes. The results with Douglas fir and nine species of pine are presented in Table I.

## SEEDS NEEDING ONLY A SOAKING IN WATER

The embryos of all the species discussed below are readily excised after soaking the seeds overnight in tap water at room temperature. Among these are some of the dormant seeds which require several months at low temperature for after-ripening as well as some of the seeds which germinate more quickly.

*Apple, pear, and European mountain ash.* The results with *Pyrus malus* L. (4 varieties), *Pyrus ussuriensis* Maxim., and *Sorbus aucuparia* L. (*Pyrus aucuparia* [L.] Ehrh.), comparing the excision method with the germination test, are shown in Tables I and III. The seeds of these three species must be after-ripened at low temperature for 6 to 12 weeks prior to germination. The embryos are readily excised when the intact seeds are soaked in water overnight, and the viability may be subsequently determined within a week. Since it was found so advantageous to keep pine seeds in moist peat for five days prior to excising, a similar experiment was tried with *Sorbus* and four varieties of apple (Table III). It was not necessary to excise some of the non-viable embryos, as they had disintegrated, and this is an advantage when the seeds to be tested contain a large proportion of non-viable seeds.

*Trumpet vine.* Two crops of *Campsis radicans* Seem. (*Bignonia radicans* Linn.) were tested. By the sixth day the cotyledons of the viable embryos were green and enlarged with considerable hypocotyl elongation, while the

dead embryos had deteriorated. The percentage viable and the germinations obtained are shown in Table I.

*Catalpa speciosa*. The excised embryos of this species develop very rapidly. Vigorous embryos develop into seedlings by the third day, while with non-viable embryos deterioration has already started. Although the hypocotyls rarely develop, the embryos low in vigor show some development such as a slight greening of the expanded cotyledons which frequently have dark spots as well as moldy areas. Embryos showing this sluggish development are still viable but lack sufficient vigor to germinate as seen in Table I. Results with vigorous embryos are also shown in the table. Germination occurs within eight weeks after planting in a 70° F. greenhouse.

*Menodora*. Within four days after excision of seeds of *M. scabra* A. Gray and *M. scoparia* Engelm. ex A. Gray, the viable embryos were green and had made considerable growth, while the non-viable embryos were discolored and deteriorating. The excision method showed 96 and 80 per cent viable (Table I). The germinations were obtained at 20° C. after mixing the intact seeds in moist peat. *M. scabra* produced 90 per cent in seven days, while *M. scoparia* gave 67 per cent germination in 14 days.

*Lyonothamnus* and *Wyethia*. In the case of these two genera (*L. floribundus* Gray and *W. scabra* Hook) optimum after-ripening conditions have not been determined, and it was not possible to get a good comparative actual germination test. However, the behavior of excised embryos was similar to all the other embryos described above.

*Eucommia ulmoides* Oliv. and *Lagerstroemia indica* L. (*L. chinensis* Lam.). Due to a very limited number of seeds only the Petri dish test was made. Here, also, the embryos developed within a week in a manner similar to other embryos. Some showed considerable vigor, others were low in vigor, while the non-viable embryos deteriorated.

#### DISCUSSION

The results in this paper and previous publications (5, 6, 7) show that the values obtained for the germinative capacity of seeds by the method of studying the behavior of the excised embryos agree closely with actual germination tests. The method is thus of practical importance in cases in which it is desirable to know the germinative capacity of dormant seeds in a shorter period of time than that required for after-ripening and germination. The method has been applied to various types of seeds, and techniques have been developed to facilitate the excising of embryos from hard-coated seeds and other types in which the excision presents a special problem. It seems safe to conclude that this quick germination test can be applied to any type of seed from which it is possible to obtain intact embryos.

The literature on various methods previously proposed for determining seed viability has been reviewed (6). Since that time an additional number of articles have been published which are briefly discussed below.

Enders and others (4) recommend Gurewitsch's dinitrobenzene method, while other workers (2, 3, 10, 11) recommend selenium or tellurium salts. Hao (8) reports that the selenium reaction is dependent on a number of factors such as respiration rate, temperature, and sulphur content of seeds. Indigo carmine and other chemicals have been used (9, 12, 13). Tanashev (14) based his method upon the detection of respiration of seeds. Zachariew (15) placed pine seeds with part of the cotyledon end of the coats removed on moist filter paper and observed that the degree of elongation and development of color was a good criterion for estimating viability. Barton (1) subjected elm seeds to optimum conditions for germination and the per cent germination thus produced agreed closely with the viability measurement obtained by observing the behavior of excised embryos on moist filter paper.

Most of the literature on this subject deals with non-dormant seeds, while the experiments reported in this article represent many types of seeds including some of the hard-coated seeds with dormant immature embryos.

#### SUMMARY

Previous work on the determination of the germinative capacity of seeds by observing the behavior of excised embryos has been extended to include 24 additional species representing 11 families. Agreement between the germinative capacity obtained by this method and actual germination tests after proper after-ripening procedures is close. The method is, therefore, applicable for a rapid determination of the germinative capacity of seeds which by ordinary methods would take impractically long.

Any difficulties arising in the use of this method are encountered in connection with the excision of the embryos. The present investigations have shown that various types of pretreatment such as acid treatment, cracking of outer coats, short period in moist peat moss, partial after-ripening of the embryos, etc., usually will overcome this difficulty in any particular seed.

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# EFFECT OF WETTING AGENTS IN INCREASING THE EFFICIENCY OF SPRAYS USED IN CONTROL OF JAPANESE BEETLE

GEORGE F. MCKENNA AND ALBERT HARTZELL

A survey was begun in 1939 to find new wetting agents to improve agricultural sprays. A list of surface active agents together with the manufacturer, type, use, and industry that employs them has been compiled by Van Antwerpen (4). Felt and Bromley (2) used Vatsol OT to increase the wetting power of sprays. Vinson and McCrory (5) recently employed Vatsol OT to increase the effectiveness of nicotine-bentonite combinations. In the present study an attempt was made to improve the effectiveness of lead arsenate for Japanese beetle (*Popillia japonica* Newm.) by means of a spreading agent rather than to find a substitute.

## MATERIALS AND METHODS

Thirty-four wetting agents were tested, 19 in liquid form and 15 in powdered form.

The substances are listed by their trade names as follows:

<i>Liquids</i>	<i>Powders</i>
Aerosol OT (10% aqueous)	Aerosol AY
Aidtex Lu-1	Aerosol MA
Bozetol	Aerosol OT
Butyl Acetyl Ricinoleate	"Alkanol" B
Butyl Oleate	Cardolite #627
Butyl Stearate	Igepal C
Daintex	Igepon T PDR
Du Pont In-438	In-181-P (Du Pont)
Ethyl Lactate	Nekal BX
Merpetine	Santomerse #1
Merpol B	Santomerse #3
Methyl Formate	Santomerse D
Methyl Lactate	Trimethyl Aminomethane
2-Methyl-2-Amino-1-Propanol	Ultrawet
Mustard Oil	Vatsol OS
Neomerpine N	
Pinespray	
Ultroil	
Yarmor 302 Pine Oil	

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The wetting agents were tested with regard to capacity to give a positive response to the following requirements:

1. Solubility or emulsibility in water (form a stable emulsion for at least 24 hours).
2. Solvency for pyrethrum resin.
3. Compatibility with lead arsenate and nicotine sulphate.
4. Non-toxicity toward sensitive plants such as tomato (*Lycopersicon esculentum* Mill. var. Bonny Best) and buckwheat (*Fagopyrum esculentum* Moench.).

Only three liquid materials fulfilled the preliminary requirements specified above. They are Ultroil, Daintex, and Pinespray. Among the powders Aerosol OT also fulfilled these requirements, except that pyrethrum resin is not soluble in an aqueous solution of it.

Ultroil is a sulphonated vegetable oil, with a pH of 6.5, a flash point of 475° F., and a boiling point of 100° C.

Daintex is listed (1) as a mixture of miscible terpene alcohols and hydrocarbons.

Pinespray<sup>1</sup> is made from a red oil converted into a semi-soap with caustic potash, but is so constructed as to carry approximately 52 per cent of Yarmor 302 Pine Oil.

Aerosol OT (dry) is the trade name for dioctyl ester of sodium sulfosuccinate (1). When available as a 10 per cent aqueous solution it is very easy to handle. Sluhan (3) has shown that molecular structure has an important bearing on the relative efficiency of these materials as detergents.

## RESULTS

1939. Experiments were made in the field with Daintex and Ultroil to determine which one was superior when used with lead arsenate in the control of Japanese beetle. The following spray mixtures were tested: (a) Ultroil 0.5 per cent, pyrethrum resin (20% total pyrethrins) 0.2 per cent, lead arsenate 0.5 per cent; (b) Ultroil 0.5 per cent, pyrethrum resin (20% total pyrethrins) 0.2 per cent, dust containing rotenone (5%) 0.5 per cent; (c) Daintex 0.5 per cent, pyrethrum resin (20% total pyrethrins) 0.2 per cent, lead arsenate 0.5 per cent; (d) Daintex 0.5 per cent, pyrethrum resin (20% total pyrethrins) 0.2 per cent, dust containing rotenone (5%) 0.5 per cent; (e) check. Pinespray was secured too late in the season to be tested in the field.

Blocks were laid out in English ivy (*Hedera helix* L.) to test wetting agents used in combination with sprays (Table I) commonly employed to control Japanese beetle adults. The treatments were applied July 3, 1939, and counts were made on July 4, 5, 6, and 7. The results are shown in Table I. The sprays containing lead arsenate gave a better control of adults than

<sup>1</sup> Private communication from Hercules Powder Co., Providence R. I.

those containing rotenone, which was also confirmed by statistical analysis. Lead arsenate sprays in addition retained their effectiveness throughout the period of the experiment (4 days), while rotenone-containing sprays showed a decrease in effectiveness with time. The foliage sprayed with rotenone-containing sprays was noticeably injured by the beetles on the last two days of the experiment. It is generally known that sunlight and moisture will destroy rotenone. The sprays containing Ultroil were superior

TABLE I  
EFFECT OF WETTING AGENTS ON SPRAYS FOR CONTROL OF JAPANESE  
BEETLE ADULTS ON ENGLISH IVY

Spray	No. beetles counted and daily % reduction in No. of beetles								Total No. of beetles counted
	July 4	% Reduc- tion	July 5	% Reduc- tion	July 6	% Reduc- tion	July 7	% Reduc- tion	
Ultroil, 0.5% Pyrethrum resin, 0.2% Lead arsenate, 0.5%	8	96.5	3	97.5	16	88.3	3	97.3	30
Ultroil, 0.5% Pyrethrum resin, 0.2% Rotenone-containing dust, 0.5%	29	83.9	29	72.4	50	63.3	65	41.0	173
Daintex, 0.5% Pyrethrum resin, 0.2% Lead arsenate, 0.5%	13	92.7	7	93.4	10	92.4	7	93.7	37
Daintex, 0.5% Pyrethrum resin, 0.2% Rotenone-containing dust, 0.5%	34	80.7	30	71.5	54	60.4	53	51.9	177
Check	176	0	105	0	136	0	110	0	517

to those containing Daintex. The superior wetting ability of Ultroil for rotenone-containing powder is offered as an explanation. However, rotenone-containing sprays must be agitated constantly to keep the particles in suspension. None of the sprays left any unsightly residue on the foliage. The foliage of the check plants was severely eaten by the beetles.

The preliminary experiments with the powdered substances showed that in this group Aerosol OT was by far the best material tested. A spray of Aerosol OT 1:1000 and lead arsenate (four pounds per 100 gal.) injured buckwheat plants slightly. Within 48 hours the plants recovered and appeared normal at the end of the experiment two days later.

The following wetting agents ranked below Aerosol OT in performance: "Alkanol" B, Nekol BX, Igepon T PDR, Santomerse #3, and a Du Pont product designated as In-181-P. Good results were secured with these

products when diluted at 1:200 to 1:500 but pyrethrum resin is not soluble in aqueous solutions of these powders. No injury occurred on tomato or buckwheat plants when these substances were used in combination with lead arsenate.

1940. Aerosol OT was the only wetting agent found in this series that would wet satisfactorily young holly plants (*Ilex opaca* Ait.). It was used at a dilution of 1:1000 in a spray of fish oil, lead arsenate, and water. The results are shown in Figure 1. The leaves shown in the figure were alike



FIGURE 1. Left: Leaf sprayed with fish oil and lead arsenate. Right: Leaf sprayed with Aerosol OT (1:1000), fish oil, and lead arsenate. Note unevenness of spray deposit on leaf at left.

except in size before the test. While Aerosol OT gave satisfactory results in laboratory tests, it is probably beyond the price range of an agricultural spray.

Three large plots of roses were sprayed to compare Ultroil and lead arsenate with two well known repellents. The sprays applied were as follows:

A. Du Pont's repellent (tetramethyl thiuram disulphide)—two and one-half pounds per 100 gallons of water for the first application and five pounds per 100 gallons for a second and third spraying.



B. Flour and lead arsenate—four pounds of wheat flour and four pounds of lead arsenate per 100 gallons of water.

C. Ultroil and lead arsenate—0.5 per cent Ultroil and four pounds of lead arsenate per 100 gallons of water.

A and B were applied to roses at the Institute on July 20, and C was applied July 22, 1940, to roses at the Boyce Thompson Arboretum, a mile distant to the east. The figures are shown in Table II.

TABLE II  
RESULTS OF SPRAYING ROSES FOR THE CONTROL OF JAPANESE BEETLE

Spray*	Date counted	No. beetles counted	No. plants counted	Av. No. beetles per bush	Weather	Feeding injury
Ultroil and lead arsenate (C)	July 25	354	302	1.2	Cloudy	None
	July 26	305	51	6.0	Sunny	None
	July 30**	228	50	4.5	Sunny	None
Tetramethyl thiuram disulphide (A)	July 22	399	50	8.0	Sunny	Severe
	July 25	485	50	9.7	Cloudy	Severe
	July 26	332	25	13.2	Sunny	Severe
Flour and lead arsenate (B)	July 22	329	50	6.6	Sunny	Slight
	July 25	281	50	5.6	Cloudy	Slight
	July 26	279	25	11.1	Sunny	Slight
Check	July 31	105	8	13.0	Sunny	Very severe
	Aug. 6	163	8	20.3	Showers	Very severe
	Aug. 7	116	8	14.5	Showers	Very severe

\* Two applications; interval, 4-5 days. For amounts of material used see text.

\*\* Roses were dusted with sulphur to control mildew about three hours before counts were made.

There is no significant difference between sprays B and C. A is definitely inferior to B in the control of the Japanese beetle. Foliage damage by beetles was quite severe in some cases with A, and it was the only case where there was any foliage injury.

The following plants have been sprayed with Ultroil 0.5 per cent and lead arsenate, four pounds per 100 gallons. This group was sprayed twice, the first spraying July 22, 1940, second spraying August 14, 1940. No injury occurred on these plants.

<i>Castanea seguinii</i> Dode.	<i>Rosa</i> sp. var. Countess Vandal
<i>Larix dahurica</i> Turcz.	Duquesade Penaranda
<i>Rosa</i> sp. var. Autumn	E. G. Hill
Betty Uprichard	Eclipse
Catalonia	Edith Nellie Perkins
Christopher Stone	Editor McFarland
Condesa de Sastago	Etoile de Hollande

<i>Rosa</i> sp. var. Federico Casas	<i>Rosa moyesii</i> Hemsl. & Wils.
Gloaming	<i>Rosa glauca</i> Pourr.
Golden Dawn	<i>Salix</i> sp.
Lady Alice Stanley	<i>Salix caprea</i> L.
Little Beauty	<i>Sorbus aria</i> Crontz var. <i>decaisneana</i>
Margaret McGredy	Rehd.
Mme. Leon Pain	<i>Ulmus</i> sp.
Mrs. A. R. Waddell	<i>Ulmus parvifolia</i> Jacq.
Mrs. E. P. Thom	<i>Ulmus pumila</i> L.
Nellie E. Hillock	<i>Vitis</i> sp.
Radiant Beauty	<i>Vitis arizonica</i> Engelm.
Rochefort	<i>Vitis pentagona</i> Diels & Gilg. var.
Sir Henry Segrave	<i>bellula</i> Rehd. & Wils.
Texas Centennial	<i>Vitis wilsonae</i> Veitch.
<i>Rosa acicularis</i> Lindl. var. <i>nipponensis</i> Hook.	<i>Vitis thunbergii</i> Sieb. & Zucc.

The foliage of the willows and elms was not materially damaged by the beetles when sprayed with Ultroil and lead arsenate. As a check there were elms and willows close by and they were severely damaged.

The following plants were sprayed once for Japanese beetle and no injury resulted from the spray:

*Aesculus hippocastanum* L.  
*Betula excelsa* Ait.  
*Betula pendula* Roth. var. *dalecarlica* Schneid.  
*Malus* sp.  
*Ulmus pumila* L. var. *pinnato-ramosa* Henry

#### DISCUSSION

Although sprays containing lead arsenate are effective in the control of the Japanese beetle they are unsightly because of the white residue. The same may be said of sprays containing wheat flour or Bordeaux. Ultroil, however, is not only colorless in solution, but when it is added to lead arsenate it makes the combined residue less visible than that of lead arsenate alone. Nevertheless, these tests show that the combination of lead arsenate and Ultroil give good control of adult Japanese beetles (Tables I and II).

Ultroil is cheap enough to be used commercially as an agricultural spray. It is very easy to handle and is not injurious to plants or man. Lead arsenate, nicotine sulphate, pyrethrum resin, rotenone-containing dust, and certain rotenone liquid extracts can be used in combination with it. Besides being a good wetting agent it is a good sticker.

Daintex will give satisfactory results and can be used with lead arsenate, nicotine sulphate, pyrethrum resin, and rotenone-containing dust. It makes a white mixture when put in water.

Pinespray is not as easy to handle as the others. It has a rather penetrating odor that may be objectionable. A cloudy solution is secured when mixed with water, but the material disperses readily in water.

#### SUMMARY

A series of 34 wetting agents in combination with lead arsenate and rotenone sprays was tested for control of Japanese beetle adults.

Ultroil reduced the visible residue left on the foliage by lead arsenate and caused no injury to 48 species and varieties of plants. When it was used in conjunction with lead arsenate in this way good control of adult Japanese beetles was obtained.

Daintex was satisfactory with the same combinations but formed a cloudy solution in water and did not spread as well as Ultroil.

Both Pinespray and Aerosol OT were satisfactory with lead arsenate spray in laboratory tests but were not tested in the field.

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# FLUCTUATIONS OF ATMOSPHERIC SULPHUR DIOXIDE

W. J. YODEN

## INTRODUCTION

Various forms of combustion, whether for heating purposes or as a part of certain industrial processes, add large quantities of sulphur dioxide to the atmosphere. Just as is the case with carbon dioxide, or water vapor, the resultant concentration of sulphur dioxide varies from place to place, and may show wide fluctuations at different times in the same place. The latter aspect of the problem is discussed in this paper.

During the course of investigations (6, 7) at this Institute concerning sulphur dioxide injury to plants, a continuous record of the concentration of this gas in the atmosphere was obtained. The apparatus and procedure are described in detail in a paper (4) by Setterstrom and Zimmerman. These authors published a summary (5) of the records for the first year. The primary interest of these workers was to ascertain whether there occurred in the atmosphere concentrations of sulphur dioxide of sufficient intensity and duration to cause detectable injury to plants. Consequently, their report (5) as well as a subsequent one by Setterstrom (3) is chiefly concerned with tabulating the date, duration, and amount whenever the observed concentration of sulphur dioxide exceeded 0.10 part per million.

The original records give a reading every 32 minutes, although, from the curves on the recorder chart, averages taken over a shorter interval may be determined. Even with the 32-minute unit these 16,000 observations for the year are far too extensive to publish in detail. They are available for examination in the Institute library. These data have been studied at considerable length, and it has been found that there is (a) a daily cycle which is different in different seasons of the year; (b) a weekly cycle; (c) an annual cycle; and (d) that the observations are correlated with wind direction and velocity, rainfall, and temperature.

## PERIODIC FLUCTUATIONS IN THE OCCURRENCE OF SULPHUR DIOXIDE

*Daily cycle.* The atmospheric concentration of  $\text{SO}_2$  throughout the course of the day often follows a most irregular course. There may be several peaks and valleys during 24 hours. The previously mentioned paper (4, Fig. 3) gives a graph for five successive days, and the report (5, Fig. 1) shows graphs for two-day periods selected at four times during the year. These specimen curves reveal the rapidity with which the concentration may change, and of course such seemingly haphazard changes obscure

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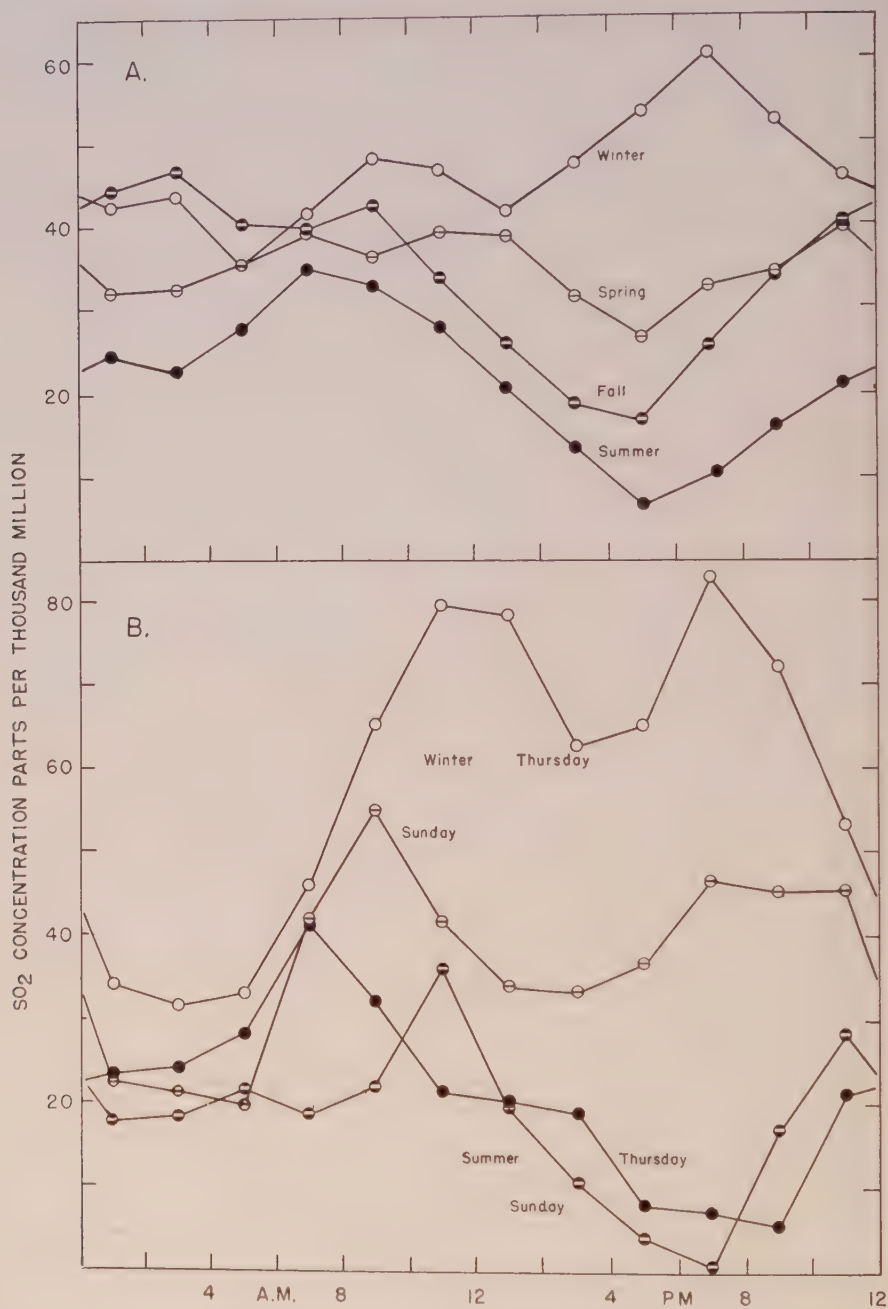


FIGURE 1. A. Daily cycle of  $\text{SO}_2$  for the four seasons. B. Sunday and Thursday cycles of  $\text{SO}_2$  for winter and summer.

any underlying cycle. In order to smooth out the daily curve the day was divided into 12 two-hour periods and the concentration obtained for each period. Instead of making a plot for an individual day a composite curve for 91 successive days was prepared. Four such curves were obtained and correspond approximately to the winter, spring, summer, and autumn seasons. Reference to Figure 1 A shows that the concentration builds up during the day in the winter season as contrasted with the other seasons which exhibit a marked drop during the afternoon. It is rather remarkable that about six o'clock in the morning the average daily concentration is nearly independent of the season.

There is, as will be seen later, a very definite weekly cycle, which suggests that the daily curve may be different for different days of the week. This was examined by collecting all the Thursdays in the winter season and contrasting the average Thursday curve with a similar curve for 13 winter Sundays. The same subdivisions of the summer were made and all four curves appear in the lower half of Figure 1. What is being tested here is not whether the Sunday and Thursday curves are superimposed, but whether they are of the same general shape. It is evident that the Sunday and Thursday curves (whether winter or summer) are more alike than the winter and summer curves (whether Sunday or Thursday). The grouping together of different weekdays to give the composite seasonal curves is thus justified.

The daily cycle at various times of the year is shown in more detail in Table I in which subdivisions of four weeks are employed. The entries in the table are in parts of  $\text{SO}_2$  per thousand million of air. A section of the table is segregated from the rest by lines which enclose those values below 30. The entries in the table are the average of approximately 100 of the 32-minute estimates. The chance variations have cancelled one another in large part and the seasonal effect is clearly brought out by the localization of values under 30 in the summer portion of the year. The hourglass shape with the constriction at six to eight o'clock is due to the relatively constant concentration at that hour throughout the year as was pointed out for the curves in Figure 1 A.

*Weekly cycle.* At this point in the work the average of all the 32-minute readings belonging to each calendar day was computed and this figure taken to represent the amount of  $\text{SO}_2$  for the day. These daily figures were then collected in groups by days of the week and by seasons. Table II, column 2, lists the averages for Sunday for the four seasons. The next six columns show the results for the other days. Regardless of the season of the year the daily average is low at the beginning of the week and builds up during the week. The averages for the whole year show Thursday and Friday to be about 50 per cent higher than Sunday and Monday. The interplay of annual and weekly cycles is shown by the fact that the two

TABLE I  
AVERAGE CONCENTRATION SO<sub>2</sub> IN PARTS PER THOUSAND MILLION OF AIR

Date and time of day	Nov. 1–Nov. 28	Nov. 29–Dec. 26	Dec. 27–Jan. 23	Jan. 24–Feb. 20	Feb. 21–Mar. 20	Mar. 21–Apr. 17	Apr. 18–May 15	May 16–June 12	June 13–July 10	July 11–Aug. 7	Aug. 8–Sept. 4	Sept. 5–Oct. 2	Oct. 3–Oct. 30
12 to 2 a.m.	46	37	41	43	35	29	29	31	15	25	24	31	79
2 to 4 a.m.	48	35	51	43	35	24	32	22	16	25	23	47	74
4 to 6 a.m.	38	34	33	46	42	29	28	21	23	32	17	44	64
6 to 8 a.m.	46	42	40	42	45	38	32	35	28	37	19	42	59
8 to 10 a.m.	50	43	58	42	35	32	36	34	24	37	35	40	53
10 to noon	40	40	65	50	26	42	40	23	18	24	32	35	33
12 to 2 p.m.	30	48	52	50	22	48	29	22	13	28	26	20	25
2 to 4 p.m.	30	63	52	50	18	31	25	16	7	12	13	17	26
4 to 6 p.m.	45	67	54	44	21	28	11	7	5	3	6	13	35
6 to 8 p.m.	53	70	66	49	32	31	19	6	9	4	10	13	58
8 to 10 p.m.	45	60	53	48	30	40	20	12	11	10	16	27	63
10 to 12 p.m.	52	46	39	50	44	40	30	15	15	20	35	33	58
Average	43	49	50	46	32	34	28	20	15	22	21	30	52

highest values, 60 and 68, in the table are for Thursday and Friday in the winter when the two cycles reinforce each other and the two lowest values, 16 and 18, are for Saturday and Sunday in the summer when both cycles are at their low points.

TABLE II  
WEEKLY CYCLE OF SO<sub>2</sub> FOR DIFFERENT SEASONS IN PARTS PER THOUSAND MILLION

Season	Day of week							Average
	S	M	T	W	T	F	S	
Nov. 1–Jan. 30	37	38	43	32	60	68	41	46
Jan. 31–May 1	20	21	36	30	41	42	48	34
May 2–July 31	18	23	21	25	22	28	16	22
Aug. 1–Oct. 30	30	36	38	30	34	41	28	34
Average	26	29	34	29	39	45	33	34

*Annual cycle.* The record for one year only has been studied, and it is not possible to show that the annual trend appears over and over in successive years as a consistent pattern. Nevertheless the average values for the different four-week periods (see Table I) show such a definite trend and lie so well along the sine-shaped curve in Figure 2 that there is little doubt that this annual cycle is a marked characteristic of the observations. It can



be imagined that the weekly cycle is superimposed on this smooth curve producing 52 pronounced ripples.

The existence of these periodic changes in the  $\text{SO}_2$  concentration, especially of different length of cycle, produces effects which may be likened to the beat heard when different tuning forks are struck. Added to these effects the vagaries of the weather induce emphatic disturbances and the net result is a series of primary observations in which the values vary in an

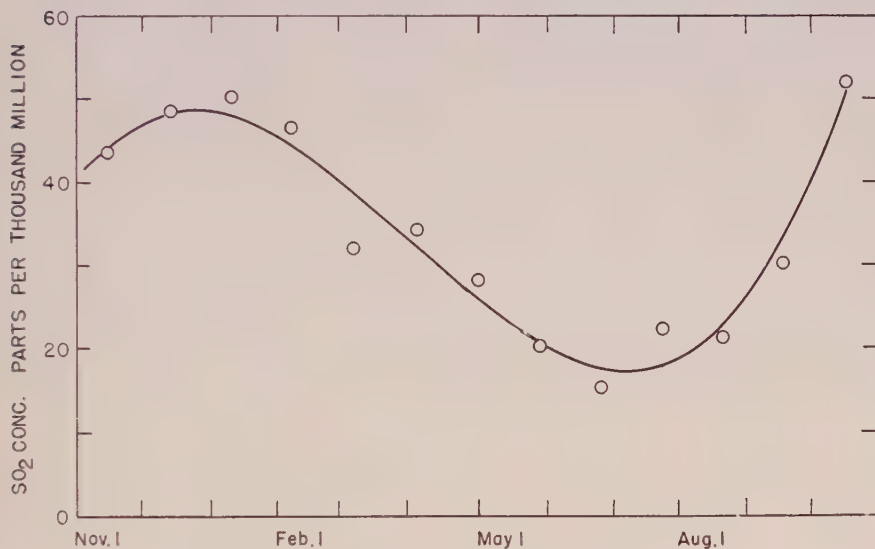


FIGURE 2. Annual cycle of  $\text{SO}_2$  concentration.

apparently capricious manner. The next section is concerned with an attempt to isolate the influence of some weather factors.

#### WEATHER FACTORS AND SULPHUR DIOXIDE CONCENTRATION

*Regression equations.* It need hardly be stressed that the weather provides variables which take on values in anything but an orderly manner. The effect of wind direction on the amount of  $\text{SO}_2$  will be best revealed if other weather factors such as total wind movement or rainfall should be held constant or nearly so. The usual advantages associated with an experiment deliberately planned to permit particular comparisons are not available here. Instead, every day provides a new combination of weather factors and the influence of any particular one may easily be obscured and lost in the midst of the simultaneous operation of concomitant factors. The best that can be done is to make a table listing the  $\text{SO}_2$  concentration day by day and tabulate alongside each value the weather data deemed significant, and then seek to establish a correlation between the weather

factors and the  $\text{SO}_2$ . It is often an advantage to establish this correlation by means of a regression equation. Consider the equation

$$Y = b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4$$

where  $b_1, b_2, \dots$  are constants and  $x_1, x_2, \dots$  are different weather factors. The weather data for the day may be substituted in the equation and the value of  $Y$  computed. If there exists a set of constants such that the computed values of  $Y$  approach more or less closely the observed values of the  $\text{SO}_2$ , it may be taken that some of these weather factors have a bearing on the amount of  $\text{SO}_2$ . A measure of the degree of association is obtained by computing the simple correlation coefficient between the observed and computed values for the  $\text{SO}_2$ . Fisher, in his text (1), illustrates this statistical procedure by associating the rainfall at a series of weather observation stations with the latitude, longitude, and altitude of the stations.

*Weather factors.* After some preliminary attempts the wind movement, rainfall, and temperature were selected as suitable factors. Certain complications were encountered in finding a suitable means of expressing wind direction and velocity. There was evidence that, when there was little wind, different directions had different effects on the  $\text{SO}_2$  concentration. On the other hand, high winds, regardless of direction, reduced the amount of  $\text{SO}_2$ . This seemed to require two variables, and consequently  $x_1$  was taken as the total wind movement for the day multiplied by the sine of the direction angle (east being taken as zero angle), and the result divided by 50 and expressed to the nearest integer. This gave northerly winds a plus sign, southerly winds a negative sign, and zero values to winds from either due east or west. The division by 50 in this case, and the choice of other factors used for subsequent variables, was dictated by the fact that the official weather data were known more accurately than the sulphur dioxide measurements. For example, on March 15, 1937 the total wind movement as given in the official record (2) was 432 miles. For the present purpose this value was rounded off to 9, i.e., all values between 426 and 474 were considered sensibly equivalent and rated as 9 units. The other wind variable,  $x_2$ , was taken as the square of the total wind movement, the result divided by 100 and rounded off to the nearest whole number. The combined rainfall for the day and the previous day expressed in tenths of an inch was designated as  $x_3$ . The inclusion of the previous day was intended to account for the presumed persistence for a time of a lowered amount of  $\text{SO}_2$  due to removal by the rain. Finally the Fahrenheit temperature, divided by 3, and rounded off to a whole number was designated as  $x_4$ . The observed values of  $y$ , tabulated in parts per million, were multiplied by 333 and rounded off to the nearest whole number. The rounding off of the data vastly lightens the numerical labor by reducing the magnitude of the numbers. It is, of course, immaterial whether the regression coefficients are subsequently adjusted to permit direct substitution of weather data in the

TABLE III  
GROUPED DATA FOR WEDNESDAY REGRESSION EQUATION

$Y$	$y$	$x_1$	$x_2$	$x_3$	$x_4$	$w$	
7.3	10	4	5	1	19	39	
11.8	12	3	5	0	14	34	
7.5	11	5	13	0	11	40	
16.9	15	-3	2	4	15	33	
6.6	1	2	8	15	13	39	
10.3	3	0	16	0	11	30	
12.4	16	1	3	5	15	40	
21.0	27	-3	4	0	9	37	
9.0	0	4	9	1	13	27	
8.3	13	6	10	0	11	40	
18.1	16	-2	3	0	13	30	
5.3	2	3	17	7	10	39	
12.1	13	5	6	0	10	34	
10.9	18	6	10	0	7	41	
10.2	13	4	8	1	12	38	
5.7	20	7	13	0	11	51	
15.2	9	2	3	0	12	26	
12.5	11	3	5	0	13	32	
14.3	5	2	8	0	9	24	
10.9	1	-3	18	1	12	29	
11.6	11	4	5	0	13	33	
12.2	22	4	4	0	13	43	
4.5	8	4	8	10	16	46	
10.8	9	-5	12	1	20	37	
2.6	1	3	13	12	15	44	
9.9	2	-4	7	13	18	36	
13.6	16	-6	8	0	21	39	
13.9	24	-5	6	0	21	46	
10.2	9	2	3	1	19	34	
11.2	15	-3	4	0	24	40	
9.2	4	-2	6	0	24	32	
10.4	19	-4	6	0	25	46	
6.0	2	1	7	0	24	34	
3.7	4	4	5	2	24	39	
6.3	5	2	3	3	24	37	
7.1	7	0	2	0	28	37	
11.1	0	-3	3	4	23	27	
8.2	2	1	3	0	24	30	
8.1	2	1	2	0	25	30	
10.3	12	-4	5	0	26	39	
3.1	12	-5	7	22	25	61	
5.6	3	1	3	2	27	36	
5.7	4	3	3	4	23	37	
6.5	6	2	1	0	27	36	
7.6	5	3	2	0	23	33	
12.6	30	-5	7	0	22	54	
15.0	8	-3	2	0	20	27	
8.4	4	3	3	2	20	32	
9.9	17	-3	4	4	24	46	
10.2	18	4	4	0	16	42	
4.1	6	-5	8	27	20	56	
10.4	3	0	6	1	19	29	
Total	506.0	506	321	328	143	933	1941
Average		9.73	.60	6.31	2.75	17.94	37.33

equation or left unaltered and the data rounded off as above before substitution.

The influence of the weather was ignored while describing the periodic cycles that  $\text{SO}_2$  exhibited with time. In the case of the cycle exhibited by the days of the week the weather would appear to be without influence since the average weather for a large number of, say, Thursdays is indistinguishable from that of any other day of the week. The daily and annual cycles, however, may be influenced by the weather since certainly the temperature and, to some extent, the wind show daily and seasonal trends. It is now desired to study the weather factors with as little interference as possible from the periodic cycles—especially the weekly one which has no conceivable association with the weather. The characteristic trend during a day will not appear if the average value for the day is taken. The weekly cycle may be circumvented by taking the data for 52 Sundays and computing a regression equation for that day of the week. If a separate equation is calculated for each day of the week the influence of the weather may be ascertained without the complication of considering the differences between week days. This leaves the annual cycle to be considered and the temperature was purposely introduced in the regression equation because it follows an annual cycle which is related to the  $\text{SO}_2$  cycle.

*Computations.* The data, after grouping, which were used to obtain the Wednesday regression equation are tabulated in Table III. The first column gives the calculated values for the  $\text{SO}_2$ , the second column the observed values, the last column under the heading  $w$  is the total of the entries in the five preceding columns and is used for checking. The sums of squares and cross products of the variables (measured from their means) are as follows:

	$y$	$x_1$	$x_2$	$x_3$	$x_4$	$w$
$y$	2764.23	-221.65	-185.69	-512.50	-411.81	
$x_1$		652.52	67.46	-246.25	-482.21	
$x_2$			833.08	189.00	-622.08	
$x_3$				1623.75	106.25	
$x_4$					1768.83	
$w$						3003.44

The  $c$  values multiplied by  $10^5$  are as follows:

$c_{11}$	$c_{12}$	$c_{13}$	$c_{14}$
207.027	25.466	24.255	63.936
	$c_{22}$	$c_{23}$	$c_{24}$
	174.776	-21.044	69.679
		$c_{33}$	$c_{34}$
		68.039	-4.877
			$c_{44}$
			98.762



Using the above results the regression coefficients are:

$$\begin{array}{cccc} b_1 & b_2 & b_3 & b_4 \\ -0.8938 & -0.5600 & -0.3433 & -0.6528 \end{array}$$

And the regression equation in which the coded values may be substituted directly is

$$Y = 26.456 - 0.894x_1 - 0.560x_2 - 0.343x_3 - 0.653x_4$$

Of the total sum of squares of deviations (2764.23) for the observed values of SO<sub>2</sub> the regression equation accounts for 746.84 leaving 2017.39 for the sum of the squares of the differences between the observed and calculated values. The correlation coefficient,  $R$ , between the observed and computed values is

$$R = \sqrt{\frac{746.84}{2764.23}} = 0.5198$$

The standard errors of the coefficients in this equation for the Wednesday observations show all of them to be significantly different from zero. The coefficients and their standard errors for the other six equations are given in Table IV which also shows the correlation coefficient for the computed and observed values.

TABLE IV  
REGRESSION EQUATION COEFFICIENTS AND STANDARD ERRORS OF COEFFICIENTS

Day	Regression coefficients				Standard error of				R
	$b_1$	$b_2$	$b_3$	$b_4$	$b_1$	$b_2$	$b_3$	$b_4$	
Sun.	-.694	.249	-.492	-.254	.403	.479	.276	.285	.355
Mon.	-.509	-.639	-.183	-.343	.397	.324	.168	.273	.454
Tues.	-1.435	-.264	-.292	-.503	.349	.314	.179	.254	.587
Wed.	-.894	-.560	-.343	-.653	.297	.274	.171	.206	.520
Thur.	-1.083	-.222	-.025	-.841	.399	.455	.230	.265	.480
Fri.	-1.300	-1.348	.259	-1.370	.507	.514	.401	.376	.510
Sat.	-.849	-1.024	-.100	-1.076	.407	.450	.408	.282	.512

Some opinion of the general agreement between the recorded observations and the calculated estimates made from the regression equation may be formed by inspecting Figure 3. It should be noted that the seasonal trend which appears in each series of observations is faithfully reflected in the computed values. The computed values fluctuate considerably less than the actual amounts. This may imply that other important factors have been neglected or that some other functions of the weather data variables might bring an improvement in the agreement. The important point is that the correlation in every case but Sunday is large enough to be significant and the effect of wind direction which was suspected in an earlier report (5) is

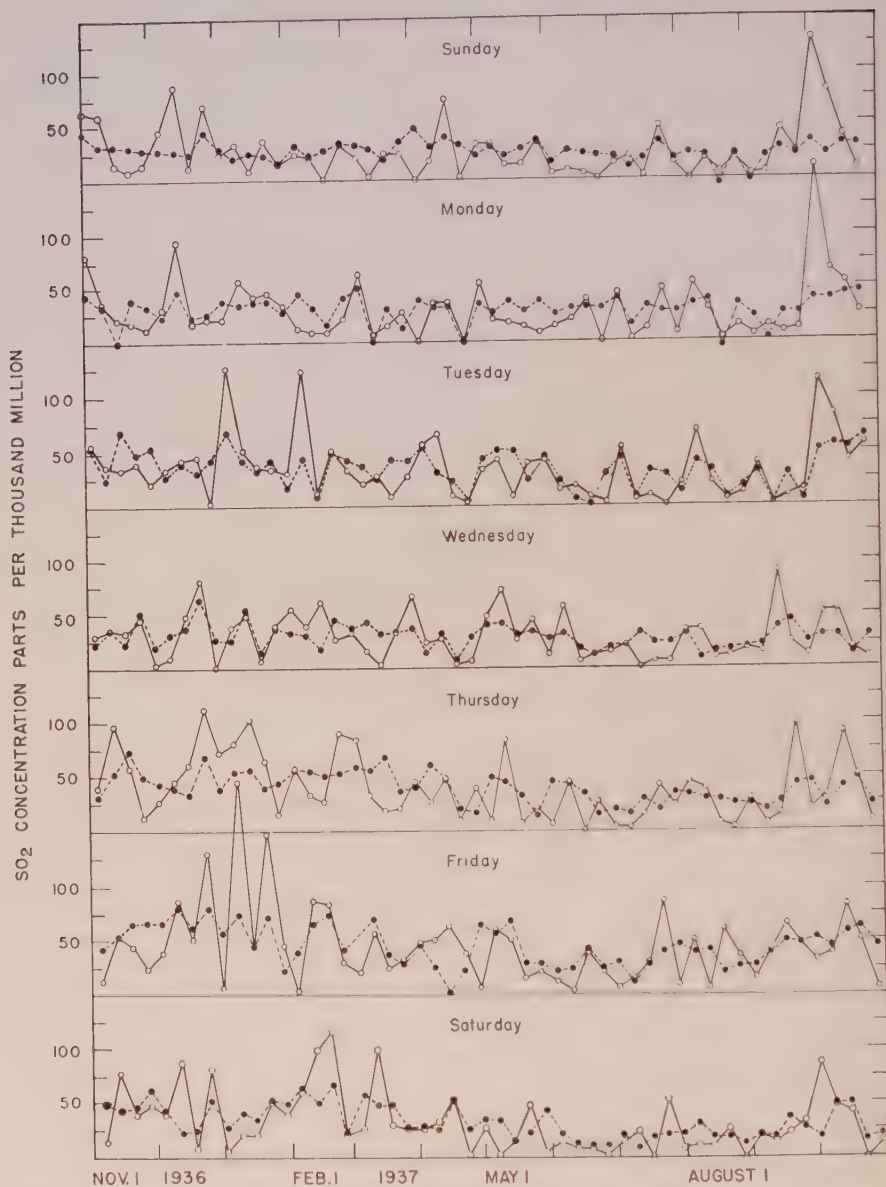


FIGURE 3. Comparison of observed concentrations with values calculated from regression equations. Solid dots represent computed values; circles the observed values.

definitely demonstrated. The correlation might be improved by omitting the measurements for the period October 1 to 15 which were not included in the report by Setterstrom and Zimmerman because of possible contamination from nearby fumigations with  $\text{SO}_2$ .

#### DISCUSSION

The resolution of the data has revealed more clearly certain relationships which were suspected of existing but by no means apparent at first glance. The more detailed analysis of the trends in the data, trends which sometimes were outweighed by opposing factors but in spite of that still operating, affords a firmer basis for the understanding of the fluctuations in the concentration of sulphur dioxide. Furthermore, the location of the Institute 15 miles north of New York City and the presence of large industrial operations to the south and east have an important bearing in the interpretation. The consistently greater concentrations prevalent in winter show how vast is the contribution of purely heating operations. The question may be raised that the wind direction, which has been shown to influence the amount of  $\text{SO}_2$ , may have prevailing directions which are associated with the season and thus be the real cause of the seasonal differences. It is possible to answer this inquiry with some assurance. The  $b_1$  regression coefficient is invariably negative and therefore northern winds have the effect of diminishing the estimate of  $\text{SO}_2$ , while south winds increase the estimate as might have been anticipated in view of the industries located south of the Institute. The prevailing winds in this region are from the northwest in winter, from the southwest in summer. These winds are actually acting in opposition to the seasonal trend, and it may be presumed that the actual seasonal contrasts may be even more striking than they appear to be from the data. There is also a prevailing daily cycle of wind direction. Northerly winds are usually found at noon and early afternoon. This may explain the pronounced drops at about noon in the spring, summer, and autumn daily curves.

The weekly cycle gives some indication of the contribution made by industrial  $\text{SO}_2$  on the assumption that industry is much less active during the week-end. There is a moderate drop in the  $\text{SO}_2$  recorded, but it is rather likely that operations of such a nature as to yield sulphur dioxide directly are of a somewhat continuous nature. There is, most certainly, a diminution in the  $\text{SO}_2$  over the week-end regardless of the season, in spite of the fact that domestic heating may well be increased at a time when people are at home in the winter.

The several sets of coefficients (Table IV) for the different days vary without apparent reason. It must be remembered that the standard errors of the coefficients are large and the variations are not evidence of inconsistency. The prevailing negative sign for the coefficients shows that northern

winds, winds of high velocity, and high temperatures and rain all tend to diminish the amount of  $\text{SO}_2$  found in this area. Gentle southerly winds, dry weather, and low temperatures produce the opposite effect. It is obvious that such a combination rarely is found because the low temperatures are found in winter when the prevailing wind is from the north and the winds are strongest.

#### SUMMARY

Examination of a continuous record of the sulphur dioxide concentration in the atmosphere shows a daily cycle which changes form with the seasons. In the winter months the concentration builds up during the day and falls off in the evening. In other seasons the drop occurs in the early afternoon and is probably the result of northerly breezes at that time of day.

There is a weekly cycle, found at all seasons, in which the concentration is low on Sunday and Monday and increases by about 50 per cent to a maximum on Thursday and Friday.

The annual cycle follows a sine-shaped curve with a minimum of 15 parts of  $\text{SO}_2$  per thousand million of air in the summer to a maximum of 50 in the winter.

The influence of the weather was brought out by deriving regression equations which show a strong correlation of sulphur dioxide concentration with wind direction and velocity, temperature, and to some extent, with rainfall.

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## RAPID TRANSMISSION OF YELLOW-RED VIROSIS IN PEACH

E. M. HILDEBRAND<sup>1</sup>

Yellow-red virosis, or "X"-disease (4, 5, 1, 2, 3), a new disease of the peach (*Prunus persica* [L.] Stokes) which also affects the chokecherry (*Prunus virginiana* L.), has offered considerable difficulty in transmission tests up to the present time. Inoculations have been slow in producing infections especially in the peach where ordinarily the trees must pass through dormancy before showing symptoms. This, together with the low and rather variable percentage of infections after passage through dormancy, has slowed progress. That difficulty should occur from interspecific transfer between peach and chokecherry was expected but not within these species. The cause for the long incubation period and the variability in percentage of infection, as yet obscure, has been sought in various ways. The way the virus is distributed in and moves from the diseased bud into healthy tissue undoubtedly depends on the growth condition of the plant most favorable to virus multiplication and movement. The discovery of a more rapid transmission method seems to be the most important challenge arising from the preliminary work. The present paper reports the results of recent transmission experiments conducted at Boyce Thompson Institute.<sup>2</sup>

### PRELIMINARY EXPERIMENTS

In order to illustrate and to emphasize the rather variable results of earlier inoculation experiments made at Ithaca, N. Y., during 1938 and 1939, a brief summary of some of the 1939 tests follows. The results of the 1938 tests have been omitted to save space.

The materials and methods employed in these earlier experiments seem worthy of brief mention. One-year-old Elberta peach trees and seedling chokecherries selected for uniformity were used exclusively. The virus was obtained from diseased plants in Columbia County, New York. In most cases the buds were inserted in the stem about six inches above soil level and held in place by nurseryman's tape slit vertically with a knife opposite the bud to prevent girdling, and removed at the end of about two weeks. In several cases the buds were placed about half-way up the stems. When employed, additional buds were distributed on the stem above the first one.

<sup>1</sup> Department of Plant Pathology, Cornell University, Ithaca, New York.

<sup>2</sup> This investigation was carried out on part time during 1939-40 at Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York, during tenure of a Guggenheim Fellowship at the Rockefeller Institute for Medical Research, Princeton, New Jersey. The author wishes to express his indebtedness and appreciation to Dr. William Crocker, Director of the Institute, for working facilities, and to members of the staff for various suggestions.

The buds from plants in an advanced stage of infection invariably were weaker than those from earlier stage material and were avoided whenever possible.

### RESULTS OF PRELIMINARY EXPERIMENTS

At the end of the second growing season the transmission efficiency from peach to peach averaged 37 per cent, ranging between 10 and 80 per cent in the several experiments, and for the other combinations from peach to chokecherry, 33; from chokecherry to peach, 27; and from chokecherry to chokecherry, 50 (Table I). Of all the inoculations made in 1939 definite symptoms showed the same season only in the case of two chokecherry

TABLE I

SUMMARY OF PRELIMINARY TRANSMISSION EXPERIMENTS DURING 1939 INVOLVING TWO BUD SOURCES AND TWO SUSCEPTS IN FOUR COMBINATIONS\*

Peach to peach		Peach to chokecherry		Chokecherry to peach		Chokecherry to chokecherry	
No. of trees	No. positive	No. of trees	No. positive	No. of trees	No. positive	No. of trees	No. positive
6	1	4	1	10	3	6	3**
12	4	4	2	2	1	6	3
20	2	4	1	10	0	4	2
10	2						
10	5						
10	6						
15	12						
10	2						
Column totals†	93	12	4	22	4	16	8

\* The great majority of these inoculation tests were made during the first half of 1939 and the final data taken about September 1, 1940 after nearly two seasons of growth.

\*\* Two plants in this experiment showed typical foliage color symptoms during 1939. In June 1940 they showed pronounced rosetting and were dead when final observations were made about September 1.

† When the data in the "column totals" were subjected to the  $\chi^2$  test for independence the results were very reasonable ( $\chi^2 = \sum \frac{d^2}{\text{exp.}} = 4.45$ ,  $n = 3$ ).

plants which had received diseased buds from chokecherry. The virus was transferred in all four possible combinations involving peach and chokecherry both as bud sources and as susceptibles. There was considerable variation between experiments but when the experiments were grouped the variation was no greater than what might be expected (see footnote, Table I). The great majority of the buds formed unions in all four combinations tested but most of them died in the two interspecific combinations. The fact that transmission was effected nearly as successfully in these latter combinations as in the others argues against the possibility that failure was due to inadequate unions, but still leaves open the possibility that one bud might contain insufficient inoculum in some cases.

Infection as influenced by the number of buds per tree was extremely variable when based on actual percentages (Table II). It would appear that a minimum of two buds should be used, but that nothing is to be gained by using more than two buds. However, only in the combination where peach

TABLE II

INFLUENCE OF NUMBER OF BUDS PER TREE ON THE PERCENTAGE OF INFECTION WITH YELLOW-RED VIROSIS AT THE END OF THE SECOND GROWING SEASON

Combination	No. of tests*	Source of inoculum	Suscept inoculated	Percentage infection when different numbers of buds were used							
				1	2	3	4	5	6	7	10
1	10	Peach	Peach	32	37	43	50	39	50	100	50
2	3	Peach	Chokecherry	0	37	50					
3	3	Chokecherry	Peach	0	25	100	50	0			
4	3	Chokecherry	Chokecherry	0	58	0	0	100			

\* The number of tests in combinations 2, 3, and 4 were probably too few to evaluate the influence of number of buds per tree on the percentage of infection.

buds were grafted on peach were the numbers of trees employed large enough to be reliable and there no important significance could be attached to using more than one bud. In the other three combinations one bud seemed inadequate to obtain transfer of a minimum amount of virus for infection, but failures also occurred in some cases when 3, 4, and even 5 buds were used. Therefore it was concluded that other factors, such as those affecting the presence of virus in a bud, proper union of tissue and survival adequate to insure transfer of virus, were probably more important than the number of buds used. No infected material thus far employed could be depended upon to transmit the virus 100 per cent. Occasional trees in the 1938 experiments failed to show symptoms at the end of one calendar year but became diseased during the second year. This delay instead of indicating an inadequate initial inoculum suggests rather that the growth condition which prevailed in these trees was unfavorable for liberation of virus from the diseased bud.

#### 1940 TRANSMISSION EXPERIMENTS

*Materials and methods.* Potted peach and chokecherry trees started from seed and grown in the greenhouse were used in all experiments. The seeds for the peach seedlings were supplied by Dr. L. O. Kunkel of the Rockefeller Institute and germinated readily after being held in moist peat at 5° C. for about 10 weeks. The chokecherry seed was gathered in New York and the seedlings started after suitable cold treatment.<sup>3</sup>

<sup>3</sup> Of several tested the best method for handling chokecherry seed consisted in removing the pulp, then layering in a moist sand and peat mixture in 6-inch pots, and storing outdoors. Seeds placed outdoors early in October gave over 60 per cent germination when brought in and tested early in February four months later.



FIGURE 1. Symptoms of yellow-red virosis on seedling peach trees on April 2, 1940 as a result of bud inoculations late in September 1939. Left to right: healthy tree, diseased tree almost dead which received 1 bud high on stem, and dead diseased tree which received 3 buds low on stem.



The virus was obtained from diseased trees in Columbia County, New York, and carried over winter in inoculated seedlings at Boyce Thompson Institute. The plants were inoculated in September and placed outdoors in October until December when they were transferred to a cold greenhouse until returned to a warm greenhouse for forcing late in January. Eight, or 50 per cent of the inoculated trees, showed symptoms by March 12. By April 2 two trees were practically dead and two others showed severe symptoms (Fig. 1). The tree to die first had been inoculated starting with 3 buds about 6 inches above soil level. The tree to die next had been inoculated with one bud near the top. Neither the number of buds nor their positions on the tree seemed of any significance in producing infection in these trees. While waiting for these trees to produce suitable budwood for transmission experiments, dormant budwood was again procured late in January from the original source and tested in a series of inoculation experiments from peach to peach. Many of these buds failed to form good unions but where good unions resulted no symptoms had appeared at the end of approximately three months. This was the usual result in previous tests. These particular trees were then cut back to the bud to stimulate new growth.

One month after the appearance of symptoms on the trees inoculated in the autumn, or on April 2, the budwood was first taken from them. Subsequently inoculum from actively diseased trees was used exclusively.

Starting on April 2 and continuing at weekly intervals for 10 weeks a variable number of peach trees ranging from 10 to 72 were inoculated by budding each week. Each tree ordinarily received one bud spaced midway on the stem. The wrapper used was a strip of "Sterilastic" bandage cut to size from a spool. A smaller number of chokecherries were included in these experiments.

When it was discovered that the earlier inoculations made about February 1 had not resulted in infection at the end of about three months, a number of methods were tested for speeding up symptoms. Among the devices used were defoliation, topping or cutting back the trees in different ways, and fertilization.

Defoliation consisted in removing all or part of the leaves on a tree and was used only in one experiment. Concerning the pruning methods: No. 1 was to cut off the stem immediately above the diseased bud; No. 2, to cut off the stem two buds or nodes above the diseased bud on a given date and two weeks later to cut all the way back to the diseased bud; No. 3, to delay till the two weeks were up before pruning and then to cut back to the diseased bud.

#### RESULTS OF 1940 TRANSMISSION EXPERIMENTS

The bud transmission tests from peach to peach during 1940 started off in conformity with previous experience by giving negative results for the

TABLE III  
YELLOW-RED VIROSIS BUD TRANSMISSION EXPERIMENTS FROM PEACH TO PEACH  
DURING 1940 AS MODIFIED BY PRUNING

Exp. No.	No. of trees	Date of budding	Pruning		Date symptoms observed*	Length in days of incubation period from		Percentage of trees diseased after	
			Date	Method		Inoculation	Pruning	2 months	5 months
1	5	Feb. 7	May 1	1	May 24	107	23	0	60
2	4	Feb. 20	—	—	—	—	—	—	—
3	5	Mar. 14	—	—	—	—	—	—	—
4	7	Apr. 2	May 1	1	May 28	56	27	100	100
	7		" 1, 15	2	" 21	49	21	100	100
	7		" 15	3	" 24	52	9	90	100
4a	7	Apr. 2	—	—	—	—	—	—	—
5	24	Apr. 10	May 1	1	May 28	48	27	60	67
	24		" 1, 15	2	" 28	48	27	62	68
	24		" 15	3	" 28	48	13	67	72
6	9	Apr. 16	May 1	1	May 28	42	27	80	89
	8		" 1, 15	2	" 28	42	27	67	100
	8		" 15	3	" 28	42	13	88	100
7	5	Apr. 18	May 1	1	May 28	40	27	100	100
	5		" 1, 15	2	" 28	40	27	100	100
	5		" 15	3	June 6	49	22	80	100
	4	Apr. 18**	" 1	1	June 6	49	36	50	50
	3		" 1, 15	2	—	—	—	0	0
	3		" 15	3	June 6	49	22	67	67
8	15	Apr. 24	May 1	1	May 28	34	27	40	60
	5		" 1, 15	2	" 28	34	27	60	60
	5		" 15	3	June 6	43	22	40	60
9	15 10	May 1	May 15	1	June 14	44	30	33	73
10	11	May 7	May 15	1	June 14	38	30	64	100
11	15	May 16	May 24	1	June 14	29	21	80	93
12	15	May 24**	May 31	1	June 21	28	21	27	33
13	10	May 31	May 6	1	?	?	—	—	80
14	10	June 6	—	—	—	—	—	—	—

\* Symptoms were considered positive when they had reached the stage shown in Figure 2.

\*\* Budwood not well developed.

current season, the inoculations made on February 7 failing to induce symptoms by May 1, approximately three months later.

When pruning was resorted to in an attempt to induce symptoms, the simple operation of cutting off the tops of inoculated plants gave surprising results, since by this means disease symptoms occurred within 30 days from the time the operation was performed even in cases where topping followed inoculation by 7 or 8 days. A glance at Table III will reveal incubation periods as low as 28 days which, so far as the writer is aware, are the short-



FIGURE 2. Symptoms of yellow-red virosis on seedling peach trees (left) 8 and (right) 7 weeks after inoculation but only 2 weeks after topping back to the diseased buds. At this early stage the leaves were turning yellow and rolling but leaf spot, shot hole, and defoliation were uncommon.

est incubation periods thus far reported for a virus disease on peach. The time between topping the plants and symptom expression ranged between 9 and 36 days and appeared somewhat independent of the period between budding and topping which ranged all the way from 7 to 84 days.

The results were recorded as positive when the symptoms approximated those illustrated in Figure 2. At the stage shown the leaves were turning yellow and rolling or curling upward in striking contrast to the healthy

leaves. The most typical symptoms observed in peach orchards (Fig. 3) — leaf spot, shot hole, and defoliation — were uncommon in the greenhouse. However, when diseased orchard trees were examined more closely leaf roll seemed more prevalent than was realized heretofore.

Defoliation alone failed to induce symptoms but when defoliated trees had leafed out again they reacted to pruning exactly the same as other inoculated trees. A companion experiment to No. 3 in Table III was to defoliate completely 10 trees and to remove the leaves only above the



FIGURE 3. Symptoms of yellow-red virosis on terminals of orchard trees. Left to right: 1, Healthy; 2, Healthy below but near tip early stage of disease has started as indicated by chlorosis and rolling or curling of leaves and one fruit mummy; 3 and 4, Advanced stage of disease with chlorosis, leaf spotting, shot hole and defoliation, rolling or curling of leaves, and fruit mummies.

diseased bud on 10 others on March 14. On April 2 while leafing out again these trees were budded and then on May 1 pruned back to the diseased bud. Subsequently they became infected 100 per cent, having the same incubation period of 56 days as shown for pruning method No. 1 in Exp. No. 4 (Table III). The length of the incubation periods from pruning were also identical for the respective pruning treatments.

The fertilization experiment seemed to demonstrate that fertilization beyond that already provided was unnecessary (Table IV). It should be



pointed out, however, that the trees were in a fine growing condition to start with. Out of 24 trees receiving no additional fertilizer 19, or 80 per cent, became diseased compared to 16, or 67 per cent, for trees receiving a complete 4-8-4 fertilizer and 19, or 80 per cent, for the trees receiving the complete fertilizer plus nitrate. Although a slightly lower percentage of trees receiving the complete 4-8-4 fertilizer became infected, the difference was not considered of any particular significance. The different pruning treatments gave identical results in amount of infection.

TABLE IV  
RESULTS OF FERTILIZATION EXPERIMENT

Pruning method*	No fertilizer**		Complete fertilizer 4-8-4		Complete fertilizer 4-8-4+nitrate		Totals	
	Trees infected		Trees infected		Trees infected		Trees infected	
	No.	%	No.	%	No.	%	No.	%
1	6	75	6	75	6	75	18	75
2	5	63	6	75	7	88	18	75
3	8	100	4	50	6	75	18	75
Column totals	19	80	16	67	19	80	54	75

\* The same three pruning methods were used here as in previous experiments.

\*\* Nitrate alone was not tested in this experiment but when used in a companion experiment no difference could be noted from the check or unfertilized trees.

In the 1940 experiments the percentage of trees diseased at the end of five months ran considerably higher than in previous years and frequently the maximum of 100 per cent infection had been attained within two months from budding. While buds from actively diseased trees were used almost exclusively, the supply of suitable inoculum ran low several times. As a consequence of using immature budwood the percentage of infections dropped very materially.

Bud inoculations in an experimental orchard in New York followed by severe pruning gave similar results to those obtained in greenhouse experiments. The inoculated branches were cut back to the diseased bud at the end of ten days and symptoms were showing on some of the varieties within five weeks from the date of inoculation. The same technique was suggested to Dr. B. L. Richards of Logan, Utah, who obtained similar results in his work on what is presumed to be the same disease in Utah. He states in correspondence that out of 40 trees budded on July 23 and then pruned severely, 100 per cent infection had resulted by the 26th of September.

Preliminary studies on heat inactivation of the virus in budwood have been very encouraging in the two experiments run thus far. Buds receiving

heat treatments at 50° C. for periods of 6, 8, 10, 12, and 15 minutes all survived but failed to transmit the disease (Fig. 4), whereas check trees receiving buds exposed at 25° C. all became diseased.

Studies to expand and clarify the results in the above experiments are now in progress.



FIGURE 4. Comparative growth on September 7 of 2 trees, one diseased and the other healthy, which had received 3 buds each on June 11 and were cut back to the top bud 10 days later. The budwood for the trees was held in water 6 minutes at (left) 25° and (right) 50° C., before budding. (Photograph printed from Kodachrome slide.)

#### DISCUSSION

The symptoms in greenhouse trees, as illustrated in Figures 1 and 2, while not identical to those usually encountered in the commercial orchard, were rather similar to those resulting from quick transmissions in an experimental orchard. The yellowing of the foliage was similar in both cases. Leaf spotting and shot hole were much less abundant on the former while leaf rolling or curling was less striking on the latter. Whereas all the 1940 greenhouse tests involved only seedlings, it was at first considered that the

use of seedlings instead of grafted trees might be partly responsible for the difference in symptom expression. However, leaf rolling or curling was equally pronounced for the rapid transmission tests in the experimental orchard on grafted varieties also where leaf spot symptoms were more pronounced. Manifestly since the elements in common were so much more in evidence than the differences, other factors such as speed of invasion by the virus and environmental differences may largely account for them. Extreme rolling of the leaves may be an expression of a more severe phase of the disease. Accompanying this condition was a tendency to hold the leaves longer. Following infection the diseased seedlings practically ceased growth and a considerable percentage died during the summer. This year the leaf rolling symptom appeared to be more abundant in diseased orchard trees than formerly (Fig. 3), perhaps because the writer became more observant from contact with the greenhouse experiments.

The results of inoculations using chokecherry plants, or chokecherry buds on peach, were much less striking than with peach buds on peach, both the percentage of infected plants being considerably lower and the incubation periods longer; this was especially true in tests with chokecherry as the host plant. The chokecherry had a tendency to grow rapidly for a while and then stop activity for the remainder of the season, a very undesirable feature.

The significance of this report rests mainly on the more rapid transmission of the disease over previous experience where extremely slow transmission was the rule. Instead of having the longest incubation period of any known peach virus, yellow-red virosis now appears to have the shortest. It is possible that by using similar methods other viruses of the peach, as well as of other woody plants, may respond more quickly to inoculation. The fact that orchard inoculations both in New York and Utah confirm the greenhouse results seems to leave little doubt of the efficacy of the method.

#### SUMMARY

Yellow-red virosis, or "X"-disease, has been transmitted by budding from peach to peach with the production of symptoms within one month's time. The simple device of cutting off the tops of the inoculated plants apparently liberated the virus from the diseased buds and speeded its invasion of the plants. Such rapid transmission of this disease will greatly facilitate studies with this virus. A similar technique may possibly be applicable to the study of viruses in other plants.

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